True Blood – Understanding the impact of reduced oxygen carrying capacity using a physiological, haematological and metabolomics perspective.

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Author's Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

I declare that all my original research for this study as reported in this thesis was undertaken by me during my enrolment for the degree of Doctor of Philosophy

In addition, I claim the majority of authorship for each article presented in this thesis. In doing so, I declare that the co-authors as recorded below contributed to the relevant article by way of critically analysing and commenting on that article, as necessary, so as to contribute to its interpretation. More specifically Drs Peiffer, Abbiss, Fairchild, Maker, Trengove, Gummer, Govus, Broadhurst, Thompson, Garvican-Lewis, Gore and Mr Raman contributed their expertise in relation to the analysis and report of the quantitative data present in Chapters Three, Four and Five. Each author provided their final approval of the relevant article prior to journal submission.

Nathan Lawler

Thesis Abstract

Oxygen carrying capacity has a positive association with endurance performance. As such, techniques such as altitude training can increase red cell mass (Hb_{mass}) and have been shown to improve sea level performance. In addition to haematological changes (i.e. Hb_{mass}), hypoxia can have a metabolic influence (e.g. increased oxidative and anaerobic enzyme concentration) resulting in changes to metabolites, which reflect systems level adaption to low oxygen carrying capacity environments. The use of metabolomics; therefore, represents an ideal analytical method to examine the physiological adaptations to hypoxia as this technique provides the ability to simultaneously analyse large number of metabolites present in human biological samples. The purpose of this thesis was to examine the acute (14 d) and chronic (42 d) influence of low oxygen carrying capacity on performance (Chapter Four), haematology (Chapters Three, Four and Five) and the metabolic profile (Chapter Three and Five).

Chapter Three was conducted as an observational study to examine metabolic changes associated with moderate altitude exposure (~3000 m; 196.4 ± 25.6 h) during a 14 d Live High Train Low altitude camp in 10 endurance runners (six males, four females; 29 ± 7 y). Resting whole blood samples were collected before altitude (baseline), 3 d and 14 d of altitude camp and analysed using mass spectrometry. From this data, acute metabolic profiles to altitude exposure were developed, which demonstrated significant separation between measured time points. Specifically, through mass spectrometry 36 metabolites were identified from metabolite classes relating to amino acids, glycolysis, and purine metabolism and indicate a shift in substrate utilisation (i.e. greater carbohydrate use) during acute hypoxic exposure. From canonical variate analysis, trajectories of the 36 identified metabolites were identified, with two different trajectories observed during the acute moderate hypoxic exposure. Importantly, principal components analysis highlighted greater variance in measured metabolites between-person when compared to within-person. This finding is consistent with previous literature and indicates individual variability during the adaptive response to altitude exposure.

During Chapter Four and Five, Australian Red Cross blood donation (~470 mL) was used to reduce the oxygen carrying capacity of 13 male participants, who were then monitored over a 42 d period. Measures of haemoglobin concentration and haematocrit % were obtained before blood removal (Baseline) and at seven time points (24 h, 7 d, 14 d, 21 d, 28 d, 35 d and 42 d) following blood removal. Additionally, exercise tests were conducted to assess fitness ($\dot{V}O_{2max}$) and performance (4-minute self-paced cycling time trial [4MMP]) (Chapter Four and Five) and 4 mL blood samples were obtained for metabolomic analysis (Chapter Five).

The purpose of Chapter Four was to examine the impact of reduced oxygen carrying capacity on $\dot{V}O_{2max}$ and both the performance and pacing during a 4MMP. During this study, only participants that were trained cyclists (n = 7) were used for the analyses. Blood removal resulted in a maximal decrease of 5.4 % in $\dot{V}O_{2max}$ (24 h), while the average power output during the 4MMP decreased significantly at 24 h (7 ± 6%), 7 d (6 ± 8%) and 21 d (4 ± 6%) when compared with baseline values. Furthermore, the aerobic contribution during the 4MMP was significantly reduced, when compared with baseline, by 5 ± 4%, 4 ± 5% and 4 ± 10% at 24 h, 7 d and 21 d, respectively. The rate of decline in power output upon commencement of the 4MMP was significantly attenuated and was 76 ± 20%, 72 ± 24% and 75 ± 35% lower than baseline at 24 h, 21 d and 42 d, respectively. These changes were unrelated to differences in haemoglobin concentration. Findings from this study indicated that reducing oxygen carrying capacity can influence pacing and the performance of middle-distance endurance events. Specifically, it appears that changes in pacing and performance during middle-distance endurance events are related to the ability to contribute power from aerobic metabolism, as an increase in anaerobic contribution was not observed.

The focus of Chapter Five was to investigate, in addition to measures obtained in Chapter Four, the acute and chronic changes to the metabolic profile of individuals after blood donation. Untargeted metabolomic analyses was used on 4 mL whole blood samples obtained from 13 participants at baseline and at multiple time points over 42 d following the removal of some 470 mL of whole blood. Similar to findings in Chapter Four, whole blood removal resulted in a maximal decrease in $\dot{V}O_{2max}$ (-6%) and 4MMP (-7%), with both occurring at 7 d. However, with the inclusion of the additional six "untrained" participants, significant reductions in oxygen delivery ($\dot{V}O_2$) only persisted for 24 h compared to 21 d in the subset of trained males (Chapter Four). The accelerated return is likely associated with participant fitness (i.e. $\dot{V}O_{2max}$: 53.0 ± 10.9 $mL \cdot kg^{-1} \cdot min^{-1}$; (Chapter Five) vs. $\dot{V}O_{2max}$: 60.7 ± 5.5 $mL \cdot kg^{-1} \cdot min^{-1}$ (Chapter Four)) with the addition of lesser trained males seemingly diminishing the influence of oxygen delivery in the later time points. The metabolomics analysis revealed multi-factorial changes with 40 metabolites deemed significant post blood removal. Through hierarchical cluster analysis, consistent with acute findings during Chapter Three, purine metabolites were significantly elevated immediately following blood removal (acute) and remained elevated throughout the 42 d monitoring period. This finding indicates a chronic adaptive response, which may occur to enhance oxygen delivery to the periphery.

Overall, the series of studies have shown separation in plasma metabolites during acute moderate altitude exposure, which is likely linked to substrate utilisation and purine metabolism. Furthermore, decreasing oxygen carrying capacity does not appear to influence the anaerobic contribution to high-intensity middle-distance endurance exercise even with observed reductions in aerobic contribution (Chapter Four). The use of metabolomics throughout this thesis (Chapters

Three and Five) has identified purine metabolism as an important marker of adaptation to hypoxia. Further research should focus on purine metabolism as a possible robust marker of hypoxic exposure and work to identify the unknown metabolites which were shown to be significant in the multivariate models.

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Chapter Four

Lawler, N.G., Abbiss, C.R., Raman, A., Fairchild, T.F., Maker, G.L., Trengove, R.D., Peiffer, J.J. (2017) Blood removal influences pacing during a 4-minute cycling time trial. *IJSPP*. 2017:1-27 Impact factor: [3.042] Accepted.

Chapter Five

Lawler, N.G., Abbiss, C.R., Gummer, J.P.A., Raman, A., Fairchild, T.F., Maker, G.L., Broadhurst, D.I., Trengove, R.D., Peiffer, J.J. Purine metabolites are associated with the physiological response to standard blood donation. *Prepared for Journal of Science and Medicine in sport.*

Additional publications related to this thesis:

Govus, A. D., Peeling, P, Abbiss, C. R., **Lawler, N.G.,** Thompson, K., Peiffer, J.J., Swinkels, D. W., Laarakkers, C. M., Gore, C. J., Garvican-Lewis, L. A., (2015). Live high: train low - influence on resting and post-exercise hepcidin levels Scandinavian Journal of Medicine and Science in Sports, 27 (7), 704-713 2017. [3.331] Accepted.

Conference Presentations

Lawler N.G., Broadhurst D.I., Abbiss C.R., Gummer J.P.A., Fairchild T.J., Maker G.L., Trengove R.D., Peiffer J.J. (2017) How blood removal affects exercise performance and the plasma metabolome. *XIIIth Annual Conference of the Metabolomics Society,* Brisbane, Australia.

Lawler N.G., Abbiss C.R., Raman A., Fairchild T.J., Maker G.L., Trengove R.D., Peiffer J.J. (2015) The influence of blood donation on energy contribution and pacing during middle distance cycling. *XXth Annual Congress of the European College of Sports Science,* Malmo, Sweden.

List of Abbreviations

ACN: Acetonitrile

ANOVA: Analysis of variance

$CHCL_3$: Chloroform

CL: Confidence limit

CVA: Canonical variate analysis

CV1: Canonical variate one

CV2: Canonical variate two

d: Days

ESI+/-: Electrospray ionisation positive and negative

EPO: Erythropoietin

FDR: False discovery rate

GC: Gas Chromatography

[Hb]: Hamoglobin concentration

$Hb_{mass:}$ Total haemoglobin mass

Hct %: Haematocrit percentage

HIF: Hypoxia inducible factor

HILIC: Hydrophilic interaction chromatography

HMDB: Human metabolome database

HR: Heart rate

Hz: Hertz

H_2O: Water

IHE: Intermittent hypoxic exposure

IHT: Intermittent hypoxic training

LC: Liquid Chromatography

LC-HRMS: Liquid chromatography High Resolution Mass Spectrometry

LHTH: Live high: train high

LHTL: Live high: train low

MAP: Maximal aerobic power

MeOH: Methanol

MS: Mass spectrometry

NMR: Nuclear magnetic resonance

$O_{2:}$ Oxygen

$P_{an:}$ Anaerobic mechanical power

$P_{aer:}$ Aerobic mechanical power

PCA: Principle component analysis

ppm: Parts per million

QC: Quality control

QC-RSC: Quality control-robust spline correction

QQQ: Triple quadrupole

QTOF: Quadrupole time of flight

RM-ANOVA: Repeated measures analysis of variance

RPE: Rating of perceived exertion

RPM: Revolutions per minute

RSD: Relative standard deviation

SD: Standard deviation

TOF: Time of flight

UPLC: Ultra-performance liquid chromatograph

$\dot{V}O_{2max}$: Maximal oxygen consumption

W: Watts

4MMP: 4-minute mean maximal power

Chapter 1 Introduction

1.1 Background

Within endurance sport, the ability to supply the body with energy through aerobic means is essential for overall success. Indeed, the measurement of maximal aerobic capacity ($\dot{V}O_{2max}$) is highly correlated with endurance performance [1]; thus, training the aerobic system is a key focus for many endurance-based athletes [2-4]. However, when examined in a non-heterogeneous group (i.e. trained athletes; $\dot{V}O_{2max}$: >60 $mL \cdot kg^{-1} \cdot min^{-1}$), the association between aerobic capacity and endurance performance is reduced [5]. In this circumstance other physiological factors have been identified as influencing endurance performance [6] including; economy of motion [7], fractional utilisation of $\dot{V}O_{2max}$ [8] and the sustained exercise intensity at the onset of blood lactate accumulation [1, 9]. Importantly, oxygen carrying capacity has a positive association with all of the above-mentioned predictors of performance [10]; thus, techniques (e.g. altitude exposure, erythropoietin administration, blood transfusions) which increase red blood cell mass (Hb_{mass}) can improve performance [11-14].

It is well documented that altitude training can improve endurance performance [15-20] by increasing Hb_{mass} [16, 21, 22]. Indeed, altitude training methods such as living and training at natural altitudes (> 2500 m), living at altitude and training near sea-level and living within artificial altitude environments at sea-level increased both Hb_{mass} and endurance performance [23, 24]. It is possible; however, that change in Hb_{mass} is not the primary factor influencing endurance performance after altitude exposure. In well-trained athletes, Gore et al. (1997) and Levine et al. (1997) observed no change in Hb_{mass} following prolonged altitude exposure [25, 26], while others have observed only a minimal association between change in Hb_{mass} and performance after altitude training [27]. Furthermore, the individual response to altitude exposure appear to be highly variable with some individuals demonstrating increased Hb_{mass} without changes in $\dot{V}O_{2max}$ while others have shown a reduction in Hb_{mass} but an increase in $\dot{V}O_{2max}$ [28]. Despite the lack of consistency, a recent meta-analysis concluded that for every 100 h spent at moderate altitude of 3000 m an increase of 1% in Hb_{mass} should be expected [13]. In addition to haematological changes, altitude training has been shown to increase skeletal muscle buffering capacity, oxidative and anaerobic enzyme concentration and lactate concentration, and result in a shift in substrate utilisation [29-31]. From these data, it is clear that adaptation to hypoxia occurs at a systemic level; however, to the authors knowledge, no studies have attempted to examine the adaptive process to hypoxia using a whole system approach.

Demonstrating promise in human disease [32] and nutritional [33, 34] research, metabolomics is gaining interest in the field of exercise science [35]. Providing the ability simultaneously analyse large number of metabolites present in human biological samples, the use of analytical

technologies such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) enables researchers to examine physiological outcomes using a whole system approach [36]. Within exercise science, metabolomic methods have been used to examine metabolites associated with glucose, lipids and, amino acids [35, 37], and more recently to create metabolite profiles to identify sports class (i.e. power vs. endurance) and training status (i.e. national vs. elite) of athletes [38]. Non-targeted metabolomic methods in exercise science have predominately measured metabolites related to energy production [35, 39]. Importantly, Pohjanen et al. (2007) used untargeted metabolomics to examine metabolite changes following a single exercise bout, highlighting 34 significantly altered metabolites ($p < 8.1e-05$) [40]. While the identified metabolites were not unique in biochemical terms, many were unlikely to have been measured using traditional methodologies. Metabolomics therefore represents an ideal analytical method to examine physiological adaptations to hypoxia. It is likely that using this technique, a greater understanding of the system change will be possible; thereby identifying new areas of focus for research in hypoxia.

1.2 Purpose of the research

Understanding human adaptation to low oxygen carrying capacity is essential for the use of this methodology in sport. However, most research has examined the influence of low oxygen carrying capacity using a reductionist approach, focusing only on key variables (i.e. Hb_{mass}) meaning that our current understanding is limited. The use of metabolomics can provide whole system analyses to hypoxic exposure possibly identifying new areas of focus for future research. Therefore, the purpose of the thesis is to explore low oxygen carrying capacity and its influence on fitness and performance as well as describe the acute and chronic physiological and biochemical adaptions to low oxygen carrying capacity. This thesis used two models of hypoxic exposure; 1) moderate altitude and 2) whole blood donation. Through this approach, this thesis will examine the adaptation to low oxygen carrying capacity during a commonly utilised training methodology (Live High Train Low simulated altitude exposure) and during a highly controlled decrease in oxygen carrying capacity (blood donation). The purpose of the individual chapters of the thesis has been outlined below.

Chapter Three:

"Metabolomic profiling of a 14-day altitude exposure in endurance runners"

The purpose of Chapter Three was to determine the influence of 14 d of normobaric hypoxic altitude exposure at ~3000 m on the human plasma metabolite profile in well-trained endurance runners. Furthermore, to demonstrate the utility of metabolomic methods to yield insights into physiological adaptations to altitude exposure.

Chapter Four:

"Blood removal influences pacing during a 4-minute cycling time trial"

Given the importance of both aerobic and anaerobic contribution as key to pacing strategies the purpose of Chapter Four was to examine the influence of manipulating aerobic contribution following blood donation on pacing strategies, performance and the impact on anaerobic energy contribution.

Chapter Five:

"Purine metabolites are associated with the physiological response to standard blood donation"

Chapter Five investigated the metabolic changes following blood donation using an untargeted metabolomic approach to track the associated changes up to 42 d post blood donation. This study represents an extension to the work examined in Chapter Four.

1.3 Significance of the research

This research will improve our understanding of reduced oxygen carrying capacity in athletes. Little is known about biochemical adaptation during reduced oxygen carrying capacity, with many studies measuring only single metabolites, making the interpretation of adaptation difficult. Therefore, research is needed to collectively examine metabolite markers to understand the biochemical adaptation. A greater understanding of biochemical changes using metabolomic analysis will aid in explaining the between athlete variability and associated performance changes with reduced oxygen carrying capacity.

1.4 Research questions

This thesis addressed the following research questions:

Chapter Three - Metabolomic profiling of a 14-day altitude exposure in endurance runners

1. To what extent does 14 d of normobaric, simulated altitude (~3000 m) influence the metabolic profile of ten highly-trained middle distance runners?
2. Can metabolomics reveal differences in adaptive processes of individuals during 14 d altitude camp?

Chapter Four – Blood removal influences pacing during a 4-minute cycling time trial

1. Does blood donation affect pacing strategies during a self-paced 4-min cycling time trial in trained cyclists?
2. Does blood donation affect anaerobic and aerobic energy contribution during a self-paced 4-min cycling time trial in trained cyclists?

Chapter Five – Purine metabolites are associated with the physiological response to standard blood donation

1. Using an untargeted metabolomic approach, to what extent will the plasma metabolite profile change in response to blood donation?
2. Can metabolomics track metabolic disturbances immediately following and up to 42 d following blood donation?

1.5 Thesis organisation

This thesis consists of six chapters. Chapter One provides a general introduction to the thesis. Chapter Two outlines a review of literature and is separated in two parts with Part I focused on; i) outlining the influence of aerobic metabolism on athletic performance, ii) identifying key literature focusing on the impact of blood manipulation on aerobic metabolism and exercise performance and iii) introducing the role of metabolomics in the field of exercise science generally, and specifically blood manipulation. Part II of the review provides background on common techniques in metabolomics and highlights important areas of concern for the use of metabolomics in exercise and sports science research. Chapters Three, Four and Five present the experimental studies. Finally, Chapter Six consists of the thesis summary and also discusses the potential limitations and future directions as guided by the thesis.

Each experimental chapter is presented in manuscript format with its own abstract, introduction, methodology, results and discussion section. Experimental chapters appear in the format required by the individual journal in which they have been published, are currently under review, or for which they are planned to be submitted. Slight variations have been made to increase thesis consistency. Additionally, for ease of reading, a single reference style was used throughout the thesis and all references have been placed together at the end of the thesis.

Chapter 2 Review of the literature

2.1 Introduction

Historically, maximal aerobic power ($\dot{V}O_{2max}$) has been considered as an essential component of performance during elite endurance sports [41]. However other measures, including lactate and anaerobic thresholds [42], economy of motion [5] and race strategies [43] can also influence performance and in some circumstances provide better estimates of success when compared with $\dot{V}O_{2max}$ alone [5, 44]. With the exception of race strategy, oxygen carrying capacity can influence all of the above-mentioned key variables of endurance performance [10]. As such, training strategies, such as hypoxic exposure, have been used with the intent of increasing oxygen carrying capacity and therefore endurance performance [45]. However, it is possible that other physiological changes associated with hypoxic exposure could influence endurance performance in the absence of changes to oxygen carrying capacity [46, 47]. Within the current literature, studies have examined the additional changes associated with hypoxic exposure (see the review by Gore et al. 2007) [46], with very few taking a whole system approach [31, 48, 49]. With the advent of new technologies (i.e. high-resolution mass spectrometry), the field of metabolomics may allow in-depth analysis of many of the variables which change with hypoxic exposure, thereby identifying new biological pathways of interest.

The use of metabolomics within human physiology is relatively new [50]; yet, provides an exciting avenue to explore both known and unknown changes associated with hypoxic exposure. Therefore, the purpose of this literature review is to, in Part I; 1) outline the influence of aerobic metabolism on athletic performance; 2) identify key literature focusing on the impact of blood manipulation on aerobic metabolism and exercise performance; and 3) introduce the role of metabolomics in the field of exercise science but more specifically, blood manipulation. Part II of this review will provide a background on the common techniques in metabolomics and highlight important areas of concern for the use of metabolomics in exercise and sports science research.

Part I

2.2 Oxygen consumption and endurance performance

Within endurance sport, the ability to rapidly supply the body with energy via aerobic means is essential for overall success. Historically, the measurement of an individual's maximal ability to consume oxygen ($\dot{V}O_{2max}$) has been used as a predictor of endurance performance [51, 52] with strong associations observed between $\dot{V}O_{2max}$ and common endurance performance tests such as the multi-stage shuttle run (r = 0.93), and 5 and 10 km running time trials (r = -0.95 and -0.89, respectively) [53]. Additionally, possessing a higher $\dot{V}O_{2max}$ is associated with enhanced ability to recover from repeated efforts leading to greater performance and training adaptation [54]. When assessed in a homogeneous sample however, the predictive strength of $\dot{V}O_{2max}$ for endurance performance is less clear. For instance, in 19 runners with similar fitness levels ($\dot{V}O_{2max:}$ 71.70 ± 2.80 $mL.kg^{-1}.min^{-1}$), $\dot{V}O_{2max}$ explained only 1.4% of the variation in 10 km running performance [5], in stark contrast to the 79% observed in a heterogeneous sample [53]. These findings indicate that $\dot{V}O_{2max}$ alone cannot be used as a true indicator of performance. Specifically, as groups of individuals become more homogenous in fitness, other factors become more relevant to performance [5, 55, 56]. Indeed, the power output or running velocity achieved at the lactate or anaerobic thresholds are better predictors of endurance performance when compared with $\dot{V}O_{2max}$ [57-59]. Nevertheless, dependent on the level of athlete (i.e. untrained vs. trained), these thresholds can occur between 50% and 90% of $\dot{V}O_{2max}$ [9]; thus, a well-developed aerobic energy system is still essential to allow competitive success.

2.3 Performance and energy contribution

Within the literature, time trials are commonly used to assess performance [60-62]. Although evidence indicates that a constant pace should be undertaken to ensure the 'best' performance [63], this technique is rarely achieved, with most individuals adopting a positive pacing strategy [64, 65]. During exercise, the selection of a pacing strategy is influenced by both psychological [66] and physiological factors [63]; however, the psychological impact is beyond the scope of this thesis. During middle distance (i.e. events lasting 2-4 minutes) and endurance events, pacing strategies are typically characterised by a high reliance on aerobic metabolism [43, 67, 68]. Recently, studies have focused on the distribution of energy expenditure during competition [43, 60, 69], as the contribution of aerobic and anaerobic energy to power output will vary energy expenditure and the pattern of power output during the race. It has been suggested that anaerobic energy stores are likely to have greater influence on self-selected pacing. Nevertheless, it is thought that a fixed limit of anaerobic work occurs during a middle distance event, and as such, the anaerobic power is thought to be the main metabolic pathway to determining performance and pacing [60, 70].

Recently, research has sought to explore the influence of anaerobic contribution without supplementation. For example, Lima-Silva et al (2013) observed a reduction in overall performance (~11%) during a cycling time trial, following a validated protocol designed to lower muscle glycogen [71]. The authors attributed the reduction in performance to a decrease in duration of the fast start as well as a significant reduction in power output during the final stages of the trial. Similarly, Correia-Oliveira et al. (2014) observed increased performance times (432.8 ± 8.3 s) and decreased power outputs (204.9 ± 10.9 W) compared to the control (420.8 ± 6.4 s and 218.4 ± 9.3 W) following an exercise protocol designed to reduce glycogen [72]. Together these results indicate anaerobic contribution is influenced by energy substrates available during the middle distance events.

2.4 Factors which can limit oxygen consumption

Endurance sport relies on aerobic metabolism which precipitates the need to understand the complexities of this system, specifically the factors which can limit oxygen consumption. This review has identified five areas in which limitations can negatively impact performance; 1) oxygen availability, 2) pulmonary system, 3) cardiac system, 4) oxygen transport in the blood and 5) peripheral limitations. A summary in the context of elite performance is provided below.

2.4.1 Oxygen availability

Ventilation and ultimately the diffusion of oxygen into the body are primarily driven by pressure gradients. At sea level (760 mm Hg), pulmonary ventilation allows for diffusion of high concentration of oxygen into the lungs and circulation while removing carbon dioxide. Changes in the environmental availability of oxygen, through artificial means (i.e. reduced availability) or naturally occurring through reduced atmospheric pressure with altitude exposure can negatively impact $\dot{V}O_{2max}$ [73-75] and exercise performance [76, 77]. Indeed, Peltonen et al. (2001) observed a decrease in sustainable power output during a series of incremental cycling tests in elite endurance males under hypoxic (15% oxygen; 334 ± 41 W) compared with normoxic (20.9% oxygen 383 ± 46 W) and hyperoxic (32% oxygen 404 ± 58 W) conditions [78]. The authors postulated that the differences in power output between trials could be influenced by a central governor [79], where reduced cardiac output and $\dot{V}O_{2max}$ are a result of acute hypoxia. Subsequent studies have challenged the central governor theorem. Clark et al. (2007), investigated cycling performance (power output and pacing) at four simulated altitudes (200, 1,200, 2,200, 3,200 m) using a 5-minute cycling time trial. The authors reported a dose response in power output to hypoxia during the 5-minute cycling time trial. Interestingly, pacing strategy remained similar for all trials; however, it was noted that those participants starting at higher altitudes participants were observed to have reduced power in the later period of the trial, which may indicate an inappropriate pace. Similarly, Wehrlin and Hallen (2006), examined six simulated altitudes (300,

800, 1300, 1800, 2300 and 2800 m) above sea level on $\dot{V}O_{2max}$ and performance using a hypobaric chamber [76]. The authors findings demonstrated a linear decrease in $\dot{V}O_{2max}$ (66 ± 1.6 mL·kg^{-1}·min^{-1} at 300 m vs. 55 ± 1.6 mL·kg^{-1}·min^{-1} at 2800 m) and decreased arterial haemoglobin saturation (89.0 ± 2.9% at 300 m vs. 76.5 ± 4.0% at 2800 m). There appears to be an association between $\dot{V}O_{2max}$ and arterial saturation [80], where ~70% decrease in $\dot{V}O_{2max}$ is explained by the decrease in arterial saturation. The decrease in $\dot{V}O_{2max}$ at moderate altitude levels seemingly supports previous research [74, 81], where exercise-induced hypoxemia can occur in endurance trained athletes with high $\dot{V}O_{2max}$ even at sea level. Interestingly, the decrease in arterial saturation is not apparent in an untrained individual, likely because endurance trained individuals can work at the steep end of the oxyhaemoglobin curve, causing greater hypoxaemia [80].

2.4.2 Pulmonary limitations

Pulmonary ventilation (i.e. inhalation and exhalation) of atmospheric air and diffusion of oxygen into the pulmonary blood is recognised as a limitation during exercise [82]. Defined as transit time issues, reduced efficiency and extraction at the alveoli are thought to be the primary causes of pulmonary dysfunction [83, 84]. In professional athletes (77.5 mL·kg^{-1}·min^{-1}), transit time issues are more prevalent due to high cardiac outputs resulting in a high velocity of blood flow through the pulmonary circulation [84-87]. The combined effect of increased cardiac outputs and blood velocity can result in inadequate time for full saturation (i.e. <90% saturation), representing a diffusion limitation in elite athletes [88]. The specific pulmonary limitation in trained populations was illustrated by Powers et al. (1989) who showed through breathing hyperoxic gas mixture (26% O_2) could increase the alveolar arterial diffusion gradient, a known limitation during maximal exercise. This method allowed for a measurable increase in O_2 saturation from 90.6% to 95.9% as well as $\dot{V}O_{2max}$ (70.1 to 74.7 mL·kg^{-1}·min^{-1}) in professional athletes. Interestingly, no impact was observed in a trained population (56.5 mL·kg^{-1}·min^{-1}) [83], where cardiac outputs are significantly lower when compared to professional athletes [89]. Pulmonary limitations have been documented in altitude environments, where the atmospheric O_2 partial pressure is reduced, impacting the alveolar arterial diffusion gradient [73, 90]. For example, Gore et al. (1997) measured 20 healthy men (11 trained, 9 untrained) to compare mild hypobaria (MH; 50 mmHg, 580 m altitude) on $\dot{V}O_{2max}$ [81]. The authors reported a decrease in arterial saturation in both groups during maximal exercise; however, the response was greatest in the trained group (rest vs. $\dot{V}O_{2max;}$ 97.7 ± 0.1 vs. 90.4 ± 0.5 %). From these studies, evidence indicate that pulmonary diffusion does not limit $\dot{V}O_{2max}$ in individuals classified as moderately trained. However, in highly trained athletes, where cardiac output is high, pulmonary transit time for red blood cells is low during maximal intensities and can limit $\dot{V}O_{2max}$ performance.

2.4.3 Cardiac limitations

In healthy populations, the cardiovascular system is considered a limitation for maximal oxygen consumption [91, 92] and consequently endurance performance [91, 93]. Through the study of endurance training adaptations in healthy populations, 70-85% of the limitations in $\dot{V}O_{2max}$ are attributed to maximal cardiac output [91, 94-96]. As maximal heart rate remains relatively unchanged until the age of ~35 years [97], lower stroke volume has the largest impact on cardiac output [92, 98, 99]; thus, factors which either positively or negatively influence stroke volume can influence $\dot{V}O_{2max}$ and endurance performance [100]. For example, Ogawa et al. (1992) compared two cohorts which were differentiated by age (trained young men age: 28 ± 3 years vs. trained old 63 ± 4 years) on measures of $\dot{V}O_{2max}$, maximal cardiac output and stroke volume [101]. The authors demonstrated decreased stroke volume (8%), maximal cardiac output (17%) and heart rate all contributed to an age-related decline in $\dot{V}O_{2max}$ (24%). Consequently, as individuals age, both maximal heart rate and stroke volume are reduced [101, 102], accounting for the consistent age-associated decline (~10 % per decade) in $\dot{V}O_{2max}$ observed after ~30 years of age [103-105].

2.4.4 Oxygen carrying capacity

In endurance athletes, a well-developed ability to extract oxygen from the blood (see section 2.4.6) means that oxygen delivery is considered the greatest limiting factor to aerobic metabolism [106, 107]. Therefore, the oxygen carrying capacity of blood can have a profound influence on oxygen consumption [108, 109] and endurance performance [110, 111], even with optimally functioning pulmonary and cardiac systems [106-108]. For instance, through the use of phlebotomy, Ekblom et al. (1972) observed a 13% decrease in haemoglobin concentration ([Hb]), which led to a 10% decrease in $\dot{V}O_{2max}$ (4.54 to 4.09 $L.min^{-1}$) [112]. Importantly, through the sequential removal of whole blood (400, 800 and 1,200 mL), a dose response was established between the decrease in [Hb] and $\dot{V}O_{2max}$ [112]. Increasing oxygen carrying capacity can also influence endurance performance [113, 114]. In healthy individuals at altitude (10,500 ft or 3200 m), the transfusion of 1,000 mL of fresh blood immediately increased haematocrit (Hct %) by 12%, resulting in a 35% increase in exercise tolerance. However, this effect was only transient with a return to baseline after 50 d likely due to induced polycythemia, where abnormally increased level of red blood cells exist.

2.4.5 Indicators of oxygen carrying capacity

Within the literature, multiple measures of oxygen carrying capacity exist including [Hb], Hct%, oxygen saturation (SO_2), total haemoglobin mass (Hb_{mass}) and red blood cell volume (RVC). Highly utilised [115-118], [Hb] and Hct % are relatively easy to measure as they form part of common pathology tests and, along with SO_2, provide an indication of the amount of oxygen available for delivery, per unit of blood volume [119]. By definition, [Hb] refers to the

concentration of haemoglobin within the entire blood volume, whereas Hct% is the measure of the percentage of red blood cells relative to fluid volume. Both measures have evident shortcomings for determination of oxygen transport. In athletic populations, [Hb] is influenced by cell compartmental fluid shifts, caused by changes in plasma volume, which can influence the accuracy of the measurement and are especially apparent following exercise and during periods of sickness [111, 120]. During exercise, shifts in plasma volume can result in haemoconcentration (increasing [Hb] per unit of blood) or haemodilution (decreasing [Hb] per unit of blood), both of which have been suggested to result in a poor relationship between [Hb] and $\dot{V}O_{2max}$ [121]. Bejder et al. (2017) showed a 10% increase in plasma volume following a 250% increase in training load [122]. With the increased training load, plasma volume increased by 10%, consequently decreasing the concentration of [Hb] by ~6%. The authors reported the time course of recovery for both plasma volume and [Hb] concentration as 2 and 4 days respectively, following the reversion to baseline training load [122]. Similar to [Hb], Hct% is influenced by plasma fluid shifts [123]. For instance, exercise in the heat is associated with fluid loss through sweating [124] and an increase in Hct % via dehydration [125, 126]. Within the literature, both [Hb] and Hct% continue to be used, yet researchers have collectively agreed that these measures should be viewed with some caution [127]. Regardless, increased [Hb] and Hct% can increase $\dot{V}O_{2max}$ [128, 129], with Gledhill et al. (1999) proposing that $\dot{V}O_{2max}$ can increase ~1% for each 3 $g{\cdot}L^{-1}$ [Hb] [129].

2.4.5.1 Haemoglobin mass

Total haemoglobin mass (Hb_{mass}) can be measured independently of plasma volume fluctuations; thus, providing a better measure of oxygen carrying capacity when compared with [Hb] and Hct%. Indeed, a strong correlation (r = 0.97) has been reported between Hb_{mass} and $\dot{V}O_{2max}$ [12, 114, 130]. The development of the optimised carbon monoxide (CO) rebreathing method by Schmidt and Prommer [131] provides the ability to measure Hb_{mass}, and is regarded as reliable and accurate, with reported typical error (biological and technical) of 2.2% [132] over 2 days and coefficient of variation of ~2% over 100 days in recreationally active men [133]. Importantly, the CO-rebreathing method can detect changes in Hb_{mass} following whole blood removal and reinfusion of red cells using quantities of 1-2 units (450 – 900 mL) [134]. Even though CO-rebreathing is highly regarded, it does present with multiple challenges. For instance, from an athlete's perspective, breathing CO during competition periods may be viewed negatively and therefore limit the use of this technique. The technique itself is expensive and requires logistical considerations (i.e. storage of CO), which again can limit its use in many research settings. Further, some researchers have expressed doubts, whether changes in Hb_{mass} can be distinguished beyond normal human variation associated with the measure [135]. For example, Garvican et al. (2010) showed Hb_{mass} varied by 3.3% in internationally competitive female cyclists ($\dot{V}O_{2max}$ 63.3 ± 5.0 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) during a 6-month competitive cycling season [135]. The variations observed, may in part be explained by increased training load, with 10% increase

training load translating to a 1% increase in Hb_{mass} [135]. Other researchers have shown Hb_{mass} to vary through injury [136], training and detraining [130, 137] and hypoxia [17, 138]. With recognised limitations associated with the measuring of $Hb_{mass,}$ it can be concluded that other measures are needed to characterise oxygen carrying capacity changes [125] [139, 140].

2.4.6 Peripheral diffusion limitations

Within skeletal muscle, diffusion of oxygen through the capillary bed may constrain oxygen transport in some individuals, with muscle diffusion reliant on a pressure gradient (i.e. pressure difference between the surface of the red blood cell and muscle) for transport of oxygen [141]. Within the muscle cell, the number of mitochondria determine the level of adenosine triphosphate (ATP) that can be generated from oxygen setting the limit to maximal oxygen uptake at the muscle level [141]. For example, exercise training studies have increased the capacity for ATP supply [142], as well as mitochondrial volume density [143]. Increased mitochondrial density can increase the concentration of enzymes such as succinate dehydrogenase, phosphofructokinase and lactate dehydrogenase, as well as mitochondrial density can increase $\dot{V}O_{2max}$ [144, 145]. Collectively, these studies show the importance of diffusive oxygen transport on $\dot{V}O_{2max.}$

Capillaries provide the exchange of oxygen between the blood and muscles. Exercise can increase capillary density and oxygen exchange at the muscle which can enhance endurance performance [146]. Brodal et al. (1977) assessed muscle biopsies using ATPase staining for assessment of capillary density in well-trained ($\dot{V}O_{2max}$ 72.0 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) and recreationally trained ($\dot{V}O_{2max}$ 51.3 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$) men [147]. When compared to recreationally trained men, muscle capillary density per mm^2 (821 vs. 582) and fibre ratio (2.49 vs. 1.77) were significantly greater in well-trained men. It was postulated that the increase in $\dot{V}O_{2max}$ was linked to the increased number of capillaries at the muscle or improved efficiency of those the athletes already had.

2.5 Manipulations to oxygen carrying capacity

As $\dot{V}O_{2max}$ depends on cardiac output and the ability to extract oxygen from the blood (i.e. arterial–venous oxygen difference), manipulation of either component can influence $\dot{V}O_{2max}$ and likely endurance performance [10]. Plasma volume can increase $\dot{V}O_{2max}$ in untrained populations through increased stroke volume [96], however in trained populations this effect is seemingly decreased [148]. Blood supply as well as Hb_{mass} are two important components which determine the oxygen transport capacity [149]. Theoretically, a 1 g increase in Hb_{mass} should result in an increase in $\dot{V}O_{2max}$ of approximately 4 $mL{\cdot}kg^{-1}{\cdot}min^{-1}$ [130]. Even though such changes may not occur in all individuals [27], this possible advantage has led to a focus on methods to enhance oxygen transport and thus aerobic performance [150]. Within the literature, increased Hb_{mass} can

be observed in response to training [14, 130] and altitude exposure (see review by Gore et al. 2013) [13]. Endurance training can lead to an in increase in red blood cells which, can lead to an increase in blood oxygen carrying capacity [91]. Other methods, such as blood transfusion have shown to increase $\dot{V}O_{2max}$ by 5 – 10 % [12], thus researchers have sought various training modalities which may enhance oxygen carrying capacity in the blood.

2.5.1 Haematological response to exercise

Plasma represents ~55% of total blood volume and is responsive to endurance training [122] and able to influence exercise performance [151-153]. For instance, plasma volume expansion has been observed to occur both during acute exercise (14 % expansion) [154] and chronically (12-20 %) as a response to training [96]. The acute increase in plasma volume has been linked to augmentation of fluid intake, likely influenced by electrolyte imbalance, changes in thirst and enhanced activation of the renin-angiotensin-aldosterone system [110]. Prolonged training adaptations appear to elevate intravascular blood volumes and training hypovolemia [155]. In untrained populations, plasma expansion can increase $\dot{V}O_{2max}$ due to increased stroke volume [96], however, this finding is not consistent in highly trained athletes [148]. Investigations to increased plasma volume through the use of training programs have provided varying results. Some studies have reported no change in $\dot{V}O_{2max,}$ despite increased stroke volume and cardiac output, indicating the unchanged $\dot{V}O_{2max}$ is likely offset by the effects of haemodilution on oxygen transport [156, 157]. Nevertheless, plasma volume expansion has been shown to prolong submaximal exercise performance in trained individuals [158], likely due to the increase in stroke volume [159].

Changes in plasma volume occur during exercise and after training; however, maturation of erythroid precursor cells to reticulocytes transpires slowly and is reliant on several months of training for significant changes to occur [111]. The intensity of exercise is important for the release of reticulocytes, with increased reticulocytes typically occurring immediately following strenuous exercise bouts [109]. Recently, hypoxia-inducible factors (HIF) proteins have been identified as markers for activation of genes following strenuous exercise and has been shown to activate erythropoietin as well as phosphofructokinase, vascular endothelial factor and lactate dehydrogenase following regular exercise training [160].

2.5.2 Hypoxia

Hypoxia can be mediated through exposure to natural altitude, or through the use of a simulated altitude room. Hypobaric hypoxia is achieved through acescent to altitude, where there is a notable decrease in partial pressure of oxygen, lowering the absolute oxygen available in the atmosphere. At sea level, barometric pressure is 760 mmHg and, oxygen concentration are constant at 20.9%. However, at altitude, barometric pressure is lower, falling to approximately 550 mmHg at ~ 2,700 m above sea level, decreasing the partial pressure of oxygen in the blood.

Simulated hypoxia, commonly referred to as normobaric hypoxia is where there is a decrease in the percentage of oxygen in the air, without an associated decrease in barometric pressure. This type of hypoxia is caused through increased nitrogen content and is the foundation for many simulated altitude rooms [161].

Regardless of the type of hypoxia, the aim is to decrease the arterial oxygen saturation to induce physiological responses which are likely to improve performance. There is currently no preference to hypoxic stimulus (i.e. hypobaric vs. normobaric); however, hypobaric hypoxia is a more severe condition and is regarded as superior to the other when the application is to improve performance and haematological change [162]. Hypoxic exposure can provide systemic stress, resulting in the up-regulation of HIF-1α, which binds to target genes, simulating an erythropoietic response and resulting in the release of EPO [163]. Erythropoietin release appears to be governed by the level of hypoxia, and the length of exposure and at an altitude of ~ 2500 m [164]. Erythrocyte volume expansion is mediated through accumulation of HIF-1α, which appears to be intensity dependent with greater responses consistent with higher stress situations [164]. Current literature indicates acute hypoxic exposure methods are insufficient to stimulate erythropoiesis (EPO) activity with at least 3-5 d continuous hypoxic exposure necessary to stimulate the maturation of an erythroid cell and to form an immature red blood cell (i.e. reticulocyte) [165]. As such, prolonged altitude training models have been explored and will be briefly outlined below.

2.5.3 Altitude training

The use of traditional prolonged exposure to hypoxia can be linked with increased aerobic power and anaerobic metabolism [46], erythrocyte volume expansion [21] and endurance performance [166]. This type of hypoxic exposure can be achieved through the Live High-Train High (LHTH) model, where athletes live and train at moderate altitudes (1500 to 3000 m) [46]. Alternatively, those athletes who are wanting to maintain training intensity, due to the decrease in $\dot{V}O_{2max}$ of ~7% per 1,000 m [77] can use Live High-Train Low (LHTL) model, where athletes live and sleep at moderate altitudes, but train at or near sea level. In both scenarios, prolonged exposure to hypoxia results in an up-regulation of HIF-1α, activating both EPO production, leading to an increase in Hb_{mass}, potentially influencing $\dot{V}O_{2max,}$ glucose metabolism adaptions (i.e. glycolytic enzymes and glucose transporters) and causing a shift in substrate utilisation [30]. The cascade of non-haematological events will be discussed in detail in section 2.5.4.

Much of the research on LHTH has produced equivocal results, with several studies pointing towards an improvement in performance [167, 168], while several other studies have failed to show improvements in performance [22, 25]. For example, Gore et al. (1998) failed to observe changes in Hb_{mass} in world-class track cyclists following 31 d of altitude training at 2690

m, even though a 4 % improvement in post-exposure 5-min time trial performance was recorded [169]. In an observational study by Robertson et al. (2010c), no differences in performance and insignificant change in Hb_{mass} and $\dot{V}O_{2max}$ were observed [22]. It should be noted this study was conducted over 10 nights with an average of 9-10 h exposure per night which, is below the recommended 14 nights and 16 h exposure per night to elicit a response [13]. While living and training at altitude is the traditional hypoxic model, living at altitude and training as close to sea level has become the preferred method for many endurance athletes [170]. Using this hypoxic training model, athletes can maintain training intensity, yet still get the effects of altitude through sleeping at moderate altitude [25]. Therefore, LHTL offers many advantages over LHTH, with athletes being able to maintain higher training intensities.

Many studies have achieved positive influence on performance and Hb_{mass} when using the LHTL model [22, 171-173]. For example, Stray-Gunderson et al. (2001), demonstrated after 27 d of LHTL, increased $\dot{V}O_{2max}$ and Hb_{mass} in elite runners, and 3 km time trial performance improved by 1.1%. In another LHTL study, Garvican et al. (2011) examined the influence of 26 d of simulated altitude training (exposure for ~ 16 $h \cdot d^{-1}$ at 3000 m with training at 600 m) on cycling performance and observed a 4% increase in sustainable power output during a 4-min time trial, which coincided with a 5% increase in Hb_{mass} [174]. While the findings from these studies are positive, the studies which have demonstrated performance changes have recognised methodological issues, such as no control groups. Unfortunately, in these studies, it is not possible to determine whether the change in performance were due to the altitude intervention, or the training camp. Furthermore, these findings indicate that changes in performance after altitude exposure are not solely due to increased Hb_{mass}, a point noted by Garvican et al. (2011) and are likely influenced by other factors such as increased glucose metabolism or currently unknown physiological changes [174].

2.5.4 Non-haematological adaptions

In the absence of changes in Hb_{mass}, improved endurance performance observed after altitude exposure must be mediated by other mechanisms [174]. It is possible that an increase in levels of 2,3-diphosphoglycerate (2,3-DPG) within erythrocytes can decrease the affinity of haemoglobin to oxygen, shifting the oxygen dissociation curve to the right [175]. Indeed, the shift in the oxyhaemoglobin curve is more pronounced in trained populations than untrained at both sea-level and altitude environments [175]. The mechanisms behind this occurrence remain unknown. One possible explanation is that during endurance exercise, increased nucleotide degradation results in an up-regulation of 2,3-DPG [176]. In support of this hypothesis, nucleotide supplementation can increase 2,3-DPG in neonatal rats, resulting in increased delivery of oxygen to the periphery [177]. In humans, research examining nucleotide supplements is difficult due to poor absorption of nucleotides using oral administration [176]. Moreover, activation of HIF-1α [163] is associated with changes in glycolytic capacity [29, 178], mitochondrial oxidative

phosphorylation [179], H^+ buffering capacity and improved perception of physical ability [180], all which could impact endurance performance and will be discussed below.

Blood lactate is greater during sub-maximal workloads (i.e. prescribed power output 100 W) during early exposure to moderate to high altitudes [181]. However, with acclimation, blood lactate concentration can decrease with no apparent rise in $\dot{V}O_{2max}$ [181], giving rise to the term 'lactate paradox' [182]. Lundby and colleagues investigated the 'lactate paradox' phenomenon in relation to duration spent at altitude (Basecamp of Mt Everest ~5400 m). Their findings revealed acute lactate levels increased (9.9 ± 0.3 $mmol.L^{-1}$), and fell significantly following 7 days acclimatisation at 5400 m (5.6 ± 0.5 $mmol.L^{-1}$) [183]. Interestingly, lactate levels increased with altitude exposure times with the authors reporting lactate levels at 4 weeks acclimatisation (7.8 ± 1.0 $mmol.L^{-1}$) and after 6 weeks acclimatisation following a sojourn from the summit (10.4 ± 1.1 $mmol.L^{-1}$). These results indicate that the 'lactate paradox' occurs during the acute stage as shown with increased lactate concentration and adrenaline and seemingly diminished with increased altitude time [184]. Other metabolic changes associated with the 'lactate paradox' have been suggested to be related to skeletal muscle hypoxic response, where working muscles release an increased concentration of lactate into blood circulation, allowing the body to use as fuel during the initial exposure to hypoxia [185]. However, it should be noted, lactate is regarded as a precursor to other major pathways, such as gluconeogenesis, thus interpretation of lactate is complicated because it can be affected by many different variables, such as altitude, diet and stress levels [186]. Furthermore, the measure is often one given data point and is a balance between lactate production and lactate uptake and removal from the body by other tissues [187].

During exercise at altitude, carbohydrates are the preferred fuel due to the high level of ATP generated per mole of oxygen [188]. Irrespective of altitude, it is recognised that the rate of fuel use is relative to exercise intensities, which determine metabolic fuel selection [189]. An increase in carbohydrate fuel sources inhibit free fatty acid uptake and glycerol release [190]; however, carbohydrate stores are limited, particularly during altitude exposure. Greater restriction on carbohydrate storage are possibly due to increased basal metabolic rate and insufficient energy intake, which have been observed at altitude [191]. Although not preferred, the body can utilise fat and protein metabolism during strenuous periods when carbohydrate stores are inadequate [192].

At the muscle level, it is recognised that very few measurable changes occur during altitude acclimatisation, with no increase in ATP concentration, oxidative enzyme activity and liver and muscle glycogen during altitude exposure [193]. Interestingly, Edwards et al. (2010) found aerobic muscle capacity and muscle cross-sectional area decreased in response to high altitude, despite the absence of mitochondrial function changes, indicating that skeletal muscle function can be maintained [193]. Recently, using nuclear magnetic resonance (NMR), Tissot et

al. (2009) profiled blood plasma of 16 individuals following exposure to ~4300 m, revealing an increase in lactic (29%) and succinic acid (158%) concentration, indicating an altitude reactive shift towards anaerobic energy production [194]. Increased lactate concentration indicates increased anaerobic glycolysis, whereas alterations to succinate indicate modified activity of the tricarboxylic acid cycle (TCA). Collectively, increased concentration in lactate and succinate lowered levels of glutathione (35%), indicating that individuals were under oxidative stress [195]. It is clear from the literature that physiological adaptions are affected by environmental changes and further investigation into these markers could advance understanding of human physiology.

2.6 Blood donation as a model of hypoxia

The objective of both normobaric and hypobaric hypoxia is to reduce oxygen saturation level in the blood which can induce hypoxemia [196]. Comparably, a reduction in whole blood volume can induce a level of hypoxemia which can be controlled through careful extraction of known quantities of whole blood [112]. For example, removal of ~10% of total blood volume in healthy adults can decrease oxygen carrying capacity, reflected by reduced [Hb] [117] or Hb_{mass} [197], with a resulting impact on both $\dot{V}O_{2max}$ [198-200] and exercise performance [117, 201]. Donating one unit of blood (~470 mL) can result in a loss of ~ 75 g (95% CI 68-80 g) of Hb_{mass} [197]. To illustrate the relationship between Hb_{mass} and performance Balke et al. (1954), used a standard blood donation (500 mL) to show $\dot{V}O_{2max}$ could be reduced by 9 % immediately following donation and that full recovery could be achieved in as little as 3 days [202]. Subsequent studies have demonstrated a negative correlation between blood removal and $\dot{V}O_{2max}$ and, this finding has been demonstrated in a recent systematic review by Remoortel et al. (2016) where $\dot{V}O_{2max}$ was decreased at 24 and 48 hrs after blood donation (-7% (95% CI – 11% to -3%) [117]. A proportional decrease (~8%) in Hb_{mass} can occur, with Pottgiesser et al. (2008) demonstrating reduced grams of Hb_{mass} (baseline 860 ± 105 g vs. 785 ± 104 g post donation) [197].

Similar to altitude, erythropoietin concentration can increase with time during hypoxemia. For example, Meurrens et al. (2016) investigated the effects of three repeated blood donations during a 12-month period on $\dot{V}O_{2max}$ and Hb_{mass} [203]. The authors findings showed increased serum erythropoietin concentration by 50% at day 2 and 100 % at 1 week of baseline levels yet, no change was observed in the placebo group. To the authors knowledge no studies have measured changes in HIF-1α which, is known precursor for accumulation of erythropoietin when using blood donation methods to lower oxygen carrying capacity. Theoretically, under normoxic conditions (i.e. oxygen levels 21% and oxygen saturation 94 – 100 %) the level of HIF-1α is decreased which prevents accumulation of EPO; however, in a hypoxic state HIF-1α level is increased binding to target genes and stimulating erythropoiesis [204, 205]. Therefore, regulation of HIF-1α is necessary to allow for red blood cell production, resulting in an increased production of EPO [204]. Detectable changes in variables such as heart rate, cardiac output,

substrate utilisation and mitochondrial function are all likely to be involved in the red blood cell restoration process [206]. Logically, with a reduction in oxygen carrying capacity following blood removal, an increased reliance on anaerobic contribution may reveal specific metabolic adaptations, such as H^+ buffering, which has been observed during hypoxic exposure [46]. Therefore, using blood donation as an experimental model may well induce similar responses to those of hypoxic environments and improve current understanding of changes associated with decreased oxygen carrying capacity.

Part II

2.7 Metabolomics in sport

As highlighted in Part I of this review, decreasing oxygen carrying capacity, be it through reduced oxygen availability or Hb_{mass}, is associated with a range of compensatory physiological responses [30, 163]. While increased RBCs is a key response to hypoxia [130], shifts in substrate utilisation [30], as well as changes to aerobic and anaerobic enzyme concentration [46] can occur in unison. Hypoxic exposure and blood removal provide a systemic insult; thus, it is likely that several unexplored physiological changes occur during these times. Currently, the lack of understanding associated with the physiological response to decreased oxygen availability warrants additional research. The use of techniques consistent with metabolomics (Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR)) may help to better understand the physiological response to hypoxia by providing the ability to monitor systemic changes through a metabolic profile. Part II of this literature review will provide a pipeline (Fig 2-1) for the use of metabolomics in this field. The approach to metabolomics will be discussed in reference to sample type(s) and the preparation and analytical techniques used to perform metabolomics. The post-acquisition tools required to analyse the data will also be discussed, followed by the practical applications of metabolomics in the field of exercise physiology specifically about the field of sports sciences.

2.8 Metabolomics: Application to exercise physiology

Metabolites are defined as low molecular weight reactants, intermediates or products of enzyme-mediated reactions and can be influenced by both physiology and environmental factors such as altitude, exercise, diet and disease [207]. In recent years, the development of analytical techniques and bioinformatics approaches has allowed for untargeted detection of thousands of metabolites in a single biological sample. This evolving approach, known as metabolomics, is an ideal platform for discoveries, as it may provide metabolic fingerprints for altered physiological, environmental and biochemical factors. Metabolomics, a cousin to genomics, transcriptomics and proteomics, is the closest measure of the human phenotype and seeks to profile all the low molecular weight (<1.5 kDa) metabolites in a particular system (e.g. biofluids). In physiological studies, important metabolic products include amino acids, lipids, carbohydrates and nucleotides, all involved in processes required for survival [207]. These metabolites can be studied to determine changes in an individual's health, as they are considered the end product following a biological change, either at the system or environmental level. Exercise physiology is in need of a holistic approach to studying changes occurring at any given time. Until recently, biological systems were commonly viewed with a reductionist consideration (i.e. breaking down of each

component); however, with the emergence of advanced analytical technologies, biological systems are now investigated with a holistic view [208]. It is through simultaneous profiling of metabolites that metabolomics has the potential to show great benefit, as many endogenous compounds can be readily assessed by a variety of biological fluids (i.e. serum, plasma, seminal fluid, tissue and saliva), which form part of a standard collection within the field [207]. It is possible to combine the results from physiological testing and the measures taken using metabolomics to understand biological systems and move towards the systems biology approach.

2.8.1 Approaches to metabolomics

There are currently two well-defined basic approaches to metabolomics research, termed 'targeted' and 'untargeted' analysis [207]. Using mass spectrometry to develop a 'targeted' protocol/profile, specific metabolites are unambiguously identified in pilot experiments, and associated methods for their quantification are developed. The benefit of this approach is that it can yield quantitative information on hundreds of polar and lipid species in a single analysis, an attractive experimental approach. Run times can be reduced (i.e. high-throughput) through monitoring of target metabolites through isolation and fragmentation of precursor ions and isolation of subsequent product ions; a workflow known as multiple reaction monitoring (MRM) [209]. There are shortcomings to this approach: i) metabolites that are not targeted will not be detected, potentially reducing discovery opportunities; ii) major development (i.e. multiple analytical runs) is required to create a library database of targeted compounds before the technique can be used; and iii) purchasing of authentic analytical standards is expensive. Of these, the second and third remain the major impediments for many analytical laboratories.

An alternative approach to the above method is 'untargeted' analysis, or metabolic profiling, which comprises a scouting method to achieve the broadest possible metabolite coverage, typically *m/z* 50-1000, capturing up to thousands of metabolites within a biological system [210]. The advantage of this approach is that it allows for the detection of previously unpredicted metabolite perturbations associated with a certain environment or condition. The design of an untargeted method differs to a targeted one in that it aims to produce an unbiased detection of as many metabolites as possible, to highlight 'features' that can discriminate between conditions (e.g. normal compared to altered blood profile). The major benefit of untargeted analysis is that it does not require prior knowledge of biologically relevant metabolites, and is usually referred to as hypothesis generating or discovery phase experimentation [211]. However, generating a list of potential target metabolites will strengthen experimental design as well as understanding of the biological sample. One limiting factor to untargeted metabolomics, and still regarded as a significant 'bottleneck' to metabolomics research, is the ability to identify metabolites post-data acquisition. For instance, for a metabolite to be classified as 'identified', a number of orthogonal parameters such as accurate mass, isotope abundance, MS/MS spectrum

and retention time are required and should match an authentic standard run in identical analytical conditions [212, 213]. This can be time-consuming and implausible when looking at a large number of biologically important metabolites; thus, the Metabolomics Initiative Standard has proposed the use of a scoring system, to display identification confidence [212].

Robust experimental design and handling of samples is needed to ensure the best possible reproducibility. This can be achieved through standard operating procedures, which are necessary for any multi-site testing, common to the field of exercise physiology. The following sections will outline study design considerations for incorporating metabolomics into the field of exercise physiology.

2.8.2 Sample collection

A range of biological samples have been used for metabolomic studies, each providing different information. The sample type should be chosen according to availability and the specific aims of the study, with the most common types being blood [34, 214], urine [215], cerebrospinal fluid [216], saliva [217], feces [218], breath condensates and tissue extracts taken from biopsies [219]. To date, most metabolomic investigations have used blood or urine, due to the relative ease of collection and minimal invasiveness [220]. In particular, blood is classed as an 'integrative fluid', meaning that it represents the system as whole, potentially indicating variation in every tissue and organ within the body [221]. However, while biofluid samples are more easily obtained, they do not explain the pathophysiological changes that may be occurring at the cell or tissue level, which may relate to changes in physiological state. For instance, skeletal muscles play a major role in overall metabolism, being the largest insulin-sensitive organ in the human body [222]. It has therefore been suggested that muscle biopsies could reveal greater pathophysiological insights into metabolic diseases and endurance exercise, as it may link to the source of substrate utilisation. Alves et al. (2014) evaluated skeletal muscle biopsy samples before and after 1 h of exhaustive exercise to assess the extraction of metabolites from ~10 mg of muscle [219]. Using a multi-platform analytical approach (LC- and GC-MS) and principal component analysis, a clear separation was observed between exercised and non-exercised states. Levels of short- and long-chain acylcarnitines were increased, presumably a result of increased substrate utilisation of fatty acids via β-oxidation, a common metabolic pathway for transport into the mitochondria [219]. Skeletal muscle samples can provide useful information, although they do have technical and ethical challenges [223], which need to be considered when choosing sample type for a specific study.

Time intervals between collection of a biological specimen, separation (centrifugation) and freezing can impact on metabolite composition [224]. This process should be tightly regulated by standard operating procedures to reduce effects on sample quality. It has been suggested that following phlebotomy, blood plasma samples should preferably be placed on ice and separated

immediately in a cooled centrifuge (4°C), as metabolites will continue to change with time [221]. Indeed, lactate concentration in plasma continue to increase as a result of continued metabolism at room temperature [225]. Furthermore, consideration needs to be given to the type of collection tube, as optimal time will vary depending on separation method. Practically, all samples should be separated within 2 h of phlebotomy and should be completed at the latest by 4 h [214]. Both plasma- and serum-yielding tubes are commonly used in exercise physiology for many biochemical and clinical tests and as such, clotting and anti-coagulant reagents are necessary. Tubes for plasma collection contain reagents such as lithium heparin, sodium citrate and ethylenediamine-tetra-acetic acid (EDTA), all of which stop blood from coagulating before cell separation by centrifugation. While tubes for serum separation contain no introduced interferences, whole blood needs to clot at room temperature, potentially causing changes in metabolite profile [226]. Yin, Lehmann and Xu (2015) provide a summary of plasma and serum metabolic differences, with increases in amino acids, nucleotides and lipids concentration in serum compared to plasma when using clotting protocols of 30 vs. 60 min [221]. It is therefore recommended that only samples with strictly controlled clotting times be used for metabolomic analysis, as variation in metabolite profiles exist with varying lengths in clotting time [225].

Relative centrifugation force (RCF) and spinning time also need consideration, as they can influence untargeted metabolomic analysis [224]. Two routine plasma centrifugation protocols (1500 × *g*, 10 min; 3000 × *g*, 5 min) were assessed using NMR and high-resolution mass spectrometry for potential differences in biochemical signature [224]. The differences were primarily identified from lipid classes (i.e. phosphocholines and sphingomyelins). Through the use of two centrifugation protocols, small individual fold changes were observed, primarily in lipid classes (i.e. phosphocholines and sphingomyelins) and was sufficient to separate metabolite profiles using multivariate statistical models.

After the separation of cells, it is common practice for samples to be stored in freezers to stop changes in metabolites prior to extraction and analysis. Metabolites can continue to be affected by enzymes and other proteins contained within serum and plasma. Small but significant changes were observed in plasma following exposure to ambient room temperature (37°C) for 24 h [227]. Specifically, Yang et al. (2013) observed increased and decreased concentration in plasma metabolites when analysed using LC-MS/MS and principal component analysis (PCA) [227]. The endogenous metabolites most affected were broadly classified as amino acids, carnitines, lysophosphatidycholines, lysophosphatidylethanolamines, carbohydrates, organic salts, bile acids, nucleosides, hormones and choline [227]. In comparison, the authors showed time-dependent changes in blood were not observed when blood samples were maintained at 4°C. Therefore, the results of Yang et al. (2013) indicate when immediate processing cannot be performed, samples should be maintained at 4°C; however, caution must be applied to some

metabolites (i.e. amino acids and acylcarnitines) which can exhibit changes in the first 24 h at 4°C [227]. Optimal temperature and length of storage is an area that requires further investigation. Samples are commonly stored between -20°C and -80°C, however -80°C is preferred for long term storage [228].

2.8.3 Sample preparation

Once the biofluid or tissue has been collected, metabolites need to be extracted and isolated for analysis. Sample preparation is crucial in order to obtain good metabolome coverage and reproducibility within a given study. The extraction solvent used needs to be tailored to the needs of the study and the number of steps kept to a minimum to ensure 'unbiased' selection of metabolites [214]. For many untargeted metabolomic analyses, sample preparation includes a clean-up step for protein precipitation. Organic solvents such as methanol, acetonitrile, isopropanol and chloroform are the most commonly used for both extraction of metabolites and protein precipitation (i.e. sample clean-up) [229, 230]. Currently, there are well-established protocols for extraction of semi-polar and polar metabolites [214], lipids [231] and other metabolite species. However, extraction protocols may need to be reviewed depending on metabolites of interest. In a recent study, Rico et al. (2014) investigated eight extraction protocols using organic solvents (i.e. methanol and acetonitrile) for separation of polar and non-polar metabolites for metabolomic studies [230]. Reversed-phase chromatography and mass spectrometry were performed on varying solvent ratios (e.g. 2:1 and 3:1 with plasma), solid–phase extraction or a combination of both. From this study, it was recommended that acetonitrile in a 2:1 ratio with plasma (v/v) offered the best coverage of the blood metabolome and provided the cleanest extracts. This finding (e.g. solvent ratio 2:1) is important and relevant to exercise physiology, as many discovery-based studies often yield a large number of samples (>200). In these cases, it is important that samples are clean in order to reduce instrumental contamination and resultant loss of sensitivity.

Since urine is regarded as the end product of metabolism, it is considered ideal for metabolomics, with urine samples being considerably less complex than blood (i.e. fewer proteins), often not requiring metabolite extraction. However, homeostatic control of urine is inferior when compared to other biofluids, so consideration is required when analysing by metabolomics. Unlike other biological fluids, urine is comprised of waste products from the entire body and is associated with high variability attributed to concentration differences. A number of normalisation methods have been proposed and can be categorised into pre- [232], where a specific dilution or injection volume is made and post-acquisition [233] (i.e. data treatment). In LC-MS metabolomics experiments a combination of pre- and post-acquisition methods is performed, where samples are diluted pre-acquisition, followed by a post-acquisition normalisation to the concentration of creatinine. A significant shortcoming to this approach is the uniform dilution can result in less concentrated urine metabolites (i.e. failure to detect low

abundant metabolites) [234]. Despite the methods popularity, it is not the preferred in athlete populations since variability in creatinine levels can occur with increase intake of both protein and creatine. Alternatively, mathematical methods such as the probabilistic quotient normalisation have been implemented as it assumes the change in concentration of metabolites influence only specific areas of the spectra [233]. However, a significant flaw to the PQN method is normalisation only works if each of your metabolites are quite similar in response. Therefore, normalisation to specific gravity via refractometry is superior due to the technique being routinely used in exercise physiology and the ability minimise variance, ultimately improving the outcome of urinary metabolomic studies and is gaining increasing traction in urine analysis [232].

2.8.4 Analytical technologies

Currently, the human metabolome is known to consist of more than 100,000 small molecules, as documented by the Human Metabolome Database (HMDB: http://www.hmdb.ca/). These endogenous molecules, comprised of carbohydrates, fatty acids, proteins, organic acids, nucleosides and nucleotides [235], vary substantially in polarity, size and concentration (i.e. µM, nM, pM). Metabolites range from hydrophilic, polar metabolites with low molecular weight (e.g. amino acids) to hydrophobic, non-polar metabolites with a much higher molecular weight (e.g. lipids). This diversity means that the unbiased detection, identification and quantification of the entire metabolome is difficult [236]. There is currently no single analytical approach that can detect or quantify all metabolites present in human samples. However, multiple analytical techniques have been employed to provide complementary coverage of a range of metabolites [237]. The combination of ^{1}H-nuclear magnetic resonance spectroscopy (NMR) with mass spectrometry (MS) has proven to be particularly effective at increasing metabolite coverage, through specificity, sensitivity and dynamic range [238, 239]. The combination of NMR and MS technologies has been central to biomarker discovery [240] and the investigation of metabolites within biological fluids such as urine, blood (plasma and serum) and saliva.

^{1}H-Nuclear magnetic resonance spectroscopy is an analytical platform that allows for the reliable detection and quantification of a wide range of metabolites in biological fluids. NMR has proven to be reproducible, due to its non-destructive and non-invasive characteristics and is well suited as a diagnostic and discovery tool, as it can resolve the structure of molecules present in a sample [241]. Typically, samples analysed by NMR are in solution, however it has been reported to successfully analyse tissue samples that remain intact [242]. In both cases, this allows samples to be analysed with no or minimal processing, thus NMR is deemed a non-destructive technique. NMR allows simultaneous detection of different metabolites through magnetic properties (e.g. spin), which help to identify the type of molecule and its chemical characteristics. NMR spectra provide an insight into structural components and enable confident identification of metabolites within a sample through the monitoring of chemical shifts. While NMR spectroscopy does offer

precise structural information, allowing for relatively quick identification [243], it has a relatively low sensitivity related to magnetic strength, as the bigger the magnet, the greater number of metabolites detected, typically ~100 metabolites in human samples [235]. This is a disadvantage when compared to mass spectrometry, as it only allows for the detection of high abundance metabolites (typically > 100 nM to 1 μM), thus profiling of the blood metabolome becomes limited, as many metabolites are present in low abundance.

Mass spectrometry, coupled with separation techniques such as liquid (LC) or gas chromatography (GC), has a higher sensitivity than NMR and the potential to detect a substantial number of low abundance metabolites (as low as 1 pM) [207]. The initial chromatography step, coupled with extremely high resolution modern instruments, such as the Orbitrap or Quadrupole-Time-of-Flight (QTOF), means that thousands of molecular features can be resolved in a single analytical run. For chromatography, the sample will exist in either gas or liquid form (mobile phase) and is then forced through a stationary phase held within a column. The stationary phase is usually an inert substance that does not react with the mobile phase to allow for partitioning of compounds between the two phases, based on their chemical properties. Typical analytical sensitivity of MS will be in the range of fM to high aM using a triple quadrupole (QQQ) instrument, and nM using a high resolution (QTOF) instrument [244]. In mass spectrometry, molecules are charged or ionised during an ionisation process (i.e. eluent entering the ion source), and these charged molecules and their fragments are separated according to their mass–to-charge ratio (*m/z*) [207]. Similar to NMR, the mass spectrometer type (i.e. QQQ, QTOF and Orbitrap) determines the mass accuracy and resolution, which are critical for structural elucidation.

While NMR spectroscopy was the tool of choice in the early phases of metabolomics, mass spectrometry based techniques are continuing to gain popularity for metabolite profiling studies because improved analytical capabilities permit much deeper metabolite coverage compared to earlier studies (1970s) in this field [245]. Mass spectrometry is not without limitations, with metabolomic applications requiring multiple ionisation states (i.e. positive and negative) and instrumental variations occurring during experimental periods as well as the formation of instrumental artefacts (i.e. adducts) [207, 246]. Retention times and signal intensities are instrument and sample dependent, requiring standardised sample preparation steps to avoid unwanted variation. Implementation of quality control samples (QCs) or internal standards within each experimental set is common to correct for small levels of variation and to quantitatively measure technical reproducibility [247]. Despite these limitations, metabolomics employing mass spectrometry is the method of choice for those seeking low abundance metabolites or resolution on large numbers of metabolites [214, 248].

2.8.5 Data pre-treatment

A number of technical issues can be generated throughout the study pipeline (see Fig 2-1) and consequently introduce artefacts (noise and unwanted variance) into metabolomic data sets. Most artefacts relate to MS, as a great strength of NMR is the robustness and reproducibility of each sample [249]. Small scale studies ($n < 100$) allow for a single sample batch to be analysed by MS in a relatively short time. By contrast, large sample studies ($n > 100$) require more care as they can often require several days or weeks of analysis. In these circumstances, individual batches need to be limited to 50-150 samples, as signal attenuation will occur over time [250]. Regular instrument cleaning should be carried out, and it has been recommended that for every 5-8 experimental samples, one quality control (QC) sample should be run [247]. Regular QC samples allow the user to check for analytical consistency and correct for any systematic drift. QC samples can be: i) small aliquots taken from each study sample to give maximal representation; ii) commercially obtained metabolites (standards) which represent metabolites of interest; or iii) purchased human serum, processed identically to study samples. Of these three, the preference is the first, due to the cost associated with commercial serum and standards. However, quality control and implementation within metabolomic studies remains an area of interest to many, as the community is yet to decide on a single standard procedure.

Metabolites exist in a given system with a wide range of concentration. High concentration metabolites will often have higher sample variance compared to low concentration metabolites. As such, in order to equalise the "weighting" of each metabolite in an unbiased statistical model, metabolites are usually scaled to unit variance (each metabolite divided by its standard deviation) [251]. After unit variance, each metabolite may be further scaled to adjust for the difference between very high and low abundance metabolites [251]. Finally, a mathematical transformation (e.g. log transformation) may be applied to make skewed data more normally distributed.

2.8.6 Data analysis

Metabolomic studies generate extensive amounts of data in a structure that is large and very complex. Statistical methods then need to be implemented to transform data into useful biological knowledge. To take into account differences in many individual metabolites, researchers can apply a reproducibility screen using defined criteria, typically a relative standard deviation (RSD) of < 20% for UHPLC-MS and < 30% for GC-MS [214, 252]. This is a provisional univariate step to remove unreproducible metabolites, which will typically remove 10-30% of all detected metabolite features from the dataset [247]. Other univariate methods such as Student's t-test and ANOVA (or non-parametric equivalents) can be useful for establishing significant differences between individual metabolites associated with different groups however, to avoid making spurious observations due to the number of statistical repeats required, additional

measures need to be incorporated. Post-hoc statistical tests such as the Bonferroni correction and Benjamini-Hochberg False Discovery Rate procedure can compensate for multiple comparisons [253, 254]. When using untargeted metabolomics methods, it is suggested that the Benjamini-Hochberg False Discovery Rate is employed to maximise discovery of metabolites as the p value correction is weighted on ranking, whereas the Bonferroni method applies a 'harsh' correction across all p values, limiting discoveries [254]. However, a major limitation to a univariate approach is that it will treat variables independently, making it difficult to determine correlations, which are important for understanding biological progressions. Changes at the system level are often due to many metabolites, and it is these larger changes that can uncover biological differences between groups. For this reason, it is common practice to use multivariate statistics in metabolomic studies to aid in finding patterns within a complex data matrix.

Principal component analysis (PCA) is an unsupervised multivariate method, used to reveal structures within a data set by reducing its complexity into fewer dimensions. This method is completely unsupervised (i.e. no group information is given) and is ideal for untargeted metabolomics, as the outcome is to compute new variable scores based on maximum variability of the data set. Latent variables or principal components (PCs) are used to explain variability in the data with the first PC accounting for the most variability in a given direction. Subsequent PCs represent the next mathematically orthogonal direction of maximum variance that is not counted in the preceding PCs [255, 256]. Often the end result is observed clusters of biochemically similar species with associated loadings, which reflect the importance of each of the metabolites. These loadings can be used to interpret the data set due to the clustering of samples with similar biological relevance.

PCA is considered the most basic multivariate projection approach, as it is only optimised to handle natural variance from within the dataset. One significant downside to this approach is that the model may not observe less obvious differences in groups of biological samples. Popular alternatives to PCA are partial least squares discriminant analysis (PLS-DA) and orthogonal PLS-DA, which are data dimension reduction strategies also commonly used in metabolomic studies [255-257]. Unlike PCA, PLS-DA is a supervised multivariate method, containing class information used to create a model of maximum variance between samples contained in 'Class 1' and 'Class 2'. Although PLS-DA is commonly used for metabolomic modelling, it may be limited in human samples as it can fail if the number of class observations exceeds six [258]. Also, PLS-DA requires class observations to be 'close-fitting', which is often difficult in metabolomic datasets with discrimination based on statistical noise instead of biological separation.

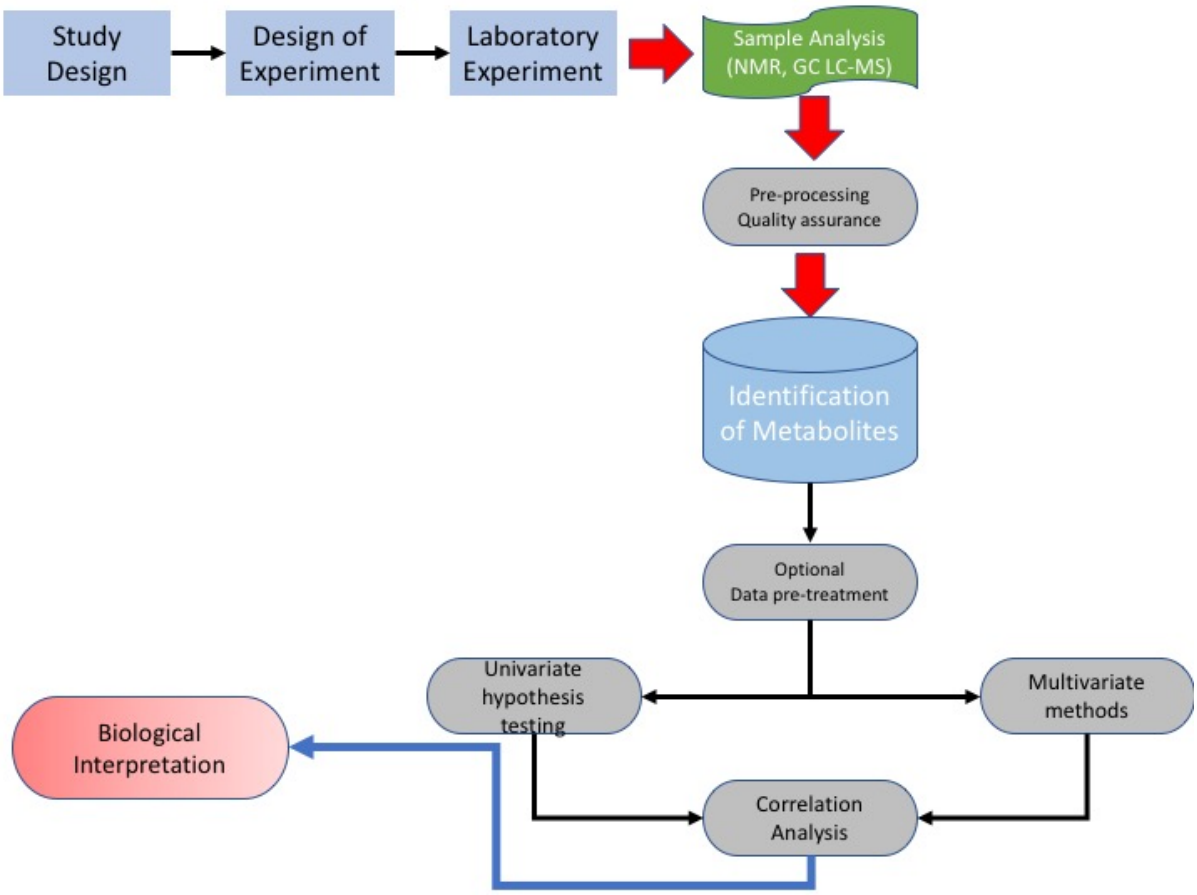

Figure 2-1 Generalised workflow for untargeted metabolomics experiments.

2.9 Critically analysing the metabolomic techniques for best practice

When interpreting the abundance of metabolomic studies, it is important to consider standardised workflow for sample processing, data acquisition, and data processing which, have not yet been achieved within the field. Metabolic profiling is typically performed using one of two contrasting analytical strategies depending on the amount of a priori biological knowledge. If very little is known then an untargeted strategy is implemented to measure a wide range of metabolites present in multiple metabolite classes, or metabolic pathways. This type of approach needs to be matched with an unselective sample preparation such as an organic solvent crash which can remove interferences such as proteins; yet, provide good solubilisation for a wide range of metabolites. Due to the chemical diversity of the metabolome, truly comprehensive metabolomic research requires LC-MS technologies due to the breadth of coverage it can provide (i.e. non-polar to polar metabolites). Of importance to discovery metabolomics is high instrument resolution, mass accuracy and measurement precision, across a full mass range. This enables metabolite identification and precise quantification of metabolite abundance. Currently, the Time of Flight and Orbitrap technologies are the gold standard for mass spectrometry metabolomics. Within this thesis untargeted metabolic profiling will be performed. Therefore, great care is needed in reducing all possible confounding variables in each of the given studies. The sensitivity

of metabolomic analysis is such that differences in metabolome can readily be detected based on inconsistent sample collection methodology, storage conditions and analytical protocols. To that end the following methods have been carefully designed and curated to avoid making false discoveries.

2.10 Metabolomics in exercise physiology research

Metabolic changes and physiological adaptations are common events in exercise physiology, constantly occurring to maintain homeostasis following exercise, environmental stimuli and stress that will innately change biochemical pathways. To date, only a handful of studies have explored the use of metabolomics in the field of exercise sciences [40, 259-265], including one of the first in the field published in 2010 [35]. Using an untargeted LC-MS metabolomics approach, the authors investigated the complex networks of events that were up-regulated following completion of single bouts of acute and prolonged exercise. In this study, subjects completed either cardiopulmonary exercise testing (Bruce protocol) or the Boston marathon (42.195 km) in which bloods were collected pre- and immediately post-event. Identified metabolites were linked to increased utilisation of fuel substrates, as measured in metabolic pathways including amino acid metabolism, glycolysis, lipolysis and adenine nucleotide metabolism. As expected, these findings supported previous literature of increased fuel substrate utilisation, such as glucogenic amino acids (i.e. alanine, threonine serine, proline). Unexpected metabolites involved in tryptophan pathway (kynurenate, quinolate and anthranilate) were found to increase following the completion of the marathon. Through the use of metabolomic analysis, this study was able to reveal additional small molecules which have not been previously detected. Furthermore, these findings provide rationale for the use of metabolomics, as some of the detected metabolites (e.g. glucose-6-phosphate and 3-phosphoglycerate) are typically only documented when using invasive techniques, such as skeletal muscle sampling [266].

Physiological changes following bouts of aerobic exercise are of interest to research fields such as sport science research and clinical populations, as an increase in cardiorespiratory fitness is indicative of health status and performance [267]. Recently, mass spectrometry has been applied to exercise intervention studies to reveal metabolic changes, which may be linked with aerobic conditioning. For example, Pohjanen et al. (2007) monitored the acute effect of strenuous cycling exercise in the blood metabolome in 24 regularly trained males and observed 34 metabolites of significant difference between pre- and post-exercise [40]. Yan et al. (2009) showed variation between senior and junior rowers through exploration of their blood metabolome, yet when using conventional biochemical parameters, no differences were observed [37]. The lack of difference observed using conventional biochemical analysis was possibly due to the homogeneity of the groups, despite participants being separated in training years (2.8 vs. 6.6 y). Interestingly, metabolomics was unable to separate senior and junior rowers prior to the

training intervention. Yet, following one and two weeks of training (technical and aerobic exercise) and strict control of diet (nutrient composition: protein 130 g; carbohydrates 700 g; fat 90 g), differences were observed between the cohorts. Contributing to the differences were alanine, glutamic acid, tyrosine and several metabolites involved in glucose metabolism. A more recent study employed LC/Orbitrap-MS based metabolomic analysis before and after submaximal cycling for a duration of 45 min, and observed significantly increased level of purine metabolites (guanine, hypoxanthine, inosine, xanthosine and deoxyinosine) in ten healthy recreationally active adults [268]. These studies have shown that metabolomics can map multiple pathways, as well as provide further physiological insight when compared to other biochemical tests.

Metabolomics has also been used to examine metabolic markers which could predict $\dot{V}O_{2max}$. Lustgarten et al. (2013) used a random forest regression to look at the association of mass spectrometry metabolites with $\dot{V}O_{2max}$ [265]. The authors identified through random forest regression (model R^2 = 0.576), metabolic markers, 4-vinylphenol sulfate, pyroxidate, 2-hydroxybutyrate showed significant associations with $\dot{V}O_{2max}$ in a cohort of 77 subjects [265]. Interestingly, when these markers were combined with blood chemistry analytes (i.e. SCOT, blood urea nitrogen) it was possible to explain 58% and 80% of $\dot{V}O_{2max}$ scores in males and females. More recently, a single urinary metabolic marker, putatively identified as oxo-aminohexanoic acid (OHA), was observed to have a strong correlation with $\dot{V}O_{2max}$ (r = 0.86) [268]. It should be noted that the biological context is yet to be determined, with the authors suggesting follow-up studies to confirm the identity of the OHA. Although the two studies are not linked, the finding of these correlations highlights the ability of metabolomics and the role it could have in assessing overall fitness in exercise physiology studies.

Considering the substantial changes associated with blood metabolites during exercise, metabolomics is regarded as a powerful tool to discover mechanisms associated with hypoxia. One of the first studies to explore hypoxia using metabolomics was conducted by Lou et al. (2014) investigating the effects of acute (2 h) exposures to simulated altitudes (12 % vs 15% vs. 21% oxygen) on the human urine metabolome [269]. The authors identified a dose dependent separation when using partial least square discriminant analysis and contributed the differences to an increase in purine markers (i.e. hypoxanthine and xanthine) and free carnitines and acetyl carnitines which may indicate a shift in energy metabolism. Importantly, the findings from this study indicate detectable metabolic adaptions occur within 2 h of exposure to altitude. More recently a metabolomics approach was adopted to assess metabolite adaption to high altitude (5300 m) and the retention of adaption follow a period of low altitude exposure (~1500m) [270]. Blood samples were collected and analysed using LC-MS during the study period. The results demonstrate an immediate metabolic adaption in the first few hours of ascending to 5000 m with increases in purine metabolites and glycolysis. Interestingly, metabolic adaptions remained following the one-week descent (~1500 m) which were consistent with improved physical

performance. Most recently Messier et al. (2017) investigated metabolic exercise performance in endurance trained individuals at sea level and at moderate terrestrial altitude (2150 m) using NMR [31]. Blood plasma analysis using NMR indicated a decrease in glucose and branched-chain amino acids after exercise at altitude, indicating a reliance on protein pathway to maintain glycaemia during exercise at moderate altitudes. Although regarded as a repeatable and reliable measure, NMR is considered complementary to MS, due to the sensitivity of the instrument which, can reduce discovery opportunities. Furthermore, it should be noted in these studies pooled QC samples were not implemented; thus, it is not possible to quantitively determine analytical precision [247, 271]. The importance of data quality checks has been stressed in the metabolomics community, illustrated through the formation of the Data Quality task group within the metabolomics society [272]. This is to ensure, researchers can monitor stability and reproducibility of the analytical process and correct small levels of drift in signal within batch and between batches [247]. Most importantly, the use of QC's can allow for the calculation of metabolites precision by using strategies for data filtering and signal correction on QC samples to reduce the potential of false discoveries. As such, the findings from these studies should be viewed cautiously as the potential for false discoveries can be increased without the use of reliable strategies.

The application of metabolomics in hypoxia-based research is relatively new and has demonstrated significant potential. Considering the stress created through altitude, it is unsurprising that altered metabolite profiles exist and demonstrate intra and inter-individual responses to altitude exposures. Considering the multifaceted nature of hypoxia, metabolomics presents as a unique technique to investigate the metabolic adaption to low oxygen carrying capacity environments.

2.11 Summary

The effect of low oxygen carrying capacity, either through removal or natural increase via hypoxic exposure, is yet to be examined beyond the typical reductionist approach, where haematological markers or individual metabolic markers have been assessed. However, with recent analytical and technological advances and improvements in study design, metabolomic studies can provide much stronger characterisation of altered physiological states, as it provides a close representation of an individual's current physiological state. Apart from the handful of abovementioned studies, there have been very few comprehensive untargeted metabolomic methods within the field of exercise physiology. A single comprehensive study by Lewis et al. (2010) conducted on the effects of exercise, has provided a backbone for metabolomic research within the field [35]. The application of newer technologies such as high-resolution mass spectrometry will allow for greater metabolite coverage to assess metabolic changes and identification of unknown metabolites, which will support exploratory studies.

The relatively new application of mass spectrometry metabolomic studies will be used to assess a broad range of metabolites taken from healthy human participants before and after blood removal (Chapter Five) and during simulated altitude exposure (Chapter Three), while the physiological impact of oxygen carrying capacity on cycling performance will be investigated in Chapter Four. This PhD will be the first study to investigate changes in haematological and physiological parameters using an untargeted metabolomics approach and high-resolution mass spectrometry in association with blood removal and altitude exposure.

Chapter 3 Metabolomic profiling of a 14-day altitude exposure in endurance runners

This chapter has been drafted to conform to the author guidelines for the Experimental Physiology journal.

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3.1 Abstract

Purpose: The purpose of this study was to determine the influence of 14 d of normobaric hypoxic altitude exposure at ~3000 m on the human plasma metabolite profile **Method:** Over 14 d, ten well trained endurance runners (six males, four females; 29 ± 7 y) lived at ~3000 m simulated altitude accumulating 196.4 ± 25.6 h of hypoxic exposure and trained at ~600 m. Resting plasma samples were collected prior to altitude (baseline), 3 d and 14 d of altitude camp. Plasma samples were analysed using liquid chromatography high resolution mass spectrometry (LC-HRMS) to construct a metabolite profile of altitude exposure. **Results:** Mass spectrometry of plasma identified 36 metabolites of which eight were statistically significant (pFDR 0.1) from baseline to either 3 d or 14 d. Specifically, changes in plasma metabolites relating to amino acid metabolism (tyrosine and proline), glycolysis (adenosine) and purine metabolism (adenosine) were observed during the altitude exposure. Principle component canonical variate analysis showed significant discrimination between group means ($p < 0.05$) with canonical variate (CV) 1 describing non-linear recovery trajectory from baseline to 3 d and then back to baseline by 14 d. Conversely, CV2 described a weaker non-recovery trajectory and increase from baseline to 3 d, with a further increase from 3 d, to 14 d. **Conclusion:** The current study demonstrates that metabolomics can be a useful tool to monitor metabolic changes associated with altitude exposure. Furthermore, it is apparent that altitude exposure alters multiple metabolic pathways, and the time course of these changes are different over the 14 d of altitude exposure.

3.2 Introduction

Endurance athletes commonly use moderate altitude exposure (2000 – 3000 m) [273] to increase their oxygen carrying capacity and subsequent athletic performance [13]. Indeed, 100 h of hypoxic exposure at ~2,200 m can increase haemoglobin mass by ~1% [13] through the upregulation of erythropoietin [164]. Under these conditions, up to a 4% increase in performance at sea level and altitude have been observed [166]. In addition to the expansion of red blood cell mass, non-haematological changes including improvements in exercise economy, skeletal muscle buffering and increased oxidative and glycolytic enzymes have all been observed following moderate altitude exposure and are associated with a post-exposure increase in performance [46]. However, improvements in performance after prolonged altitude exposure have also been observed in the absence of changes to haematological and/or non-haematological variables [273-275]. It is possible that unknown physiological adaptations following altitude exposure are responsible for the observed increase in performance or that it is not possible to accurately quantify the magnitude of change in the identified haematological and non-haematological variables [274].

Metabolomics is the comprehensive analysis of metabolites which are the end product of gene expression in a biological system [207], and may provide a viable technique to expand current understanding of the physiological adaptations associated with altitude exposure. Indeed, as oxygen is the terminal electron acceptor in the mitochondrial respiratory chain, and central energy metabolism is intricately linked to global metabolism, characterising the metabolic changes that occur during prolonged altitude exposure may identify previously unknown pathways of change that could influence exercise performance. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) is a commonly employed analytical method for the untargeted measurement of metabolites due to its ability to resolve thousands of metabolite features with a single analysis of a biological sample. This technique enables accurate mass determination (ppm error) without compromising detection sensitivity, ensuring the detection of many metabolites from complex matrices such as blood. In addition, metabolomics requires only small sample volumes (~50 μL), thus enabling the collection of biological fluids more frequently on human participants in a variety of contexts. Recently, metabolomic studies using nuclear magnetic resonance (NMR) and mass spectrometry (MS) have enabled investigation into the effects of hypoxia in human, animal and cell models [48, 49, 269, 270], revealing changes in biochemical pathways such as central carbon, purines and lipid metabolism.

Although a large volume of research has been conducted in an attempt to understand the haematological and non-haematological changes associated with altitude exposure, there are large inter- and intra-individual differences [276] in the physiological response to moderate exposure indicating that further research is necessary. The primary aim of this study was to use liquid

chromatography-high resolution mass spectrometry (LC-HRMS) analysis of human plasma samples to examine: the influence of 14 d normobaric, simulated altitude (~3000 m) on the metabolic profile of ten highly-trained middle distance runners.

3.3 Methods

3.3.1 Ethical approval

Experimental procedures were approved by the human research ethics committee of Edith Cowan University, the Australian Institute of Sport Human Ethics Committee and conducted in accordance with the Declaration of Helsinki. All individuals provided written informed consent prior to participation.

3.3.2 Participants

Ten well-trained, healthy middle distance runners (six males and four females) were recruited from the Canberra local running community [mean ± standard deviation (SD) age: 28.6 ± 6.7 y; body mass: 62.6 ± 7.6 kg; maximal oxygen consumption ($\dot{V}O_{2max}$): 65.6 ± 8.1 $mL \cdot kg^{-1} \cdot min^{-1}$] to participate in this study. Participants were included if they had at least two years of competitive running experience and trained at least three times per week. Participants were screened prior to the study for iron deficiency and excluded if deemed to be stage 2 iron deficient (serum ferritin < 20 $\mu g \cdot L^{-1}$) [277]. Participants were instructed to maintain normal training schedule during the study, but to refrain from caffeine, alcohol and strenuous physical activity on the day of blood sampling. All participants self-reported compliance with these requirements. Data for this study was collected as part of a larger observational study examining the influence of 14 d altitude exposure (~3000 m) from which changes in haemoglobin mass, haematocrit %, haemoglobin concentration and erythropoietin have been reported elsewhere [16].

3.3.3 Altitude exposure

Participants were exposed to 14 consecutive days of normobaric hypoxia (~3000 m) as part of an 'altitude training camp'. The hypoxic environment was maintained within a purpose-built altitude house facility using nitrogen dilution (Kinetic Performance Technology, Canberra, Australia). The goal of the training camp was to expose participants to at least 200 h [13] of hypoxic exposure (e.g. 14 h d^{-1}). Participants maintained their normal training schedules during the 14 d experiment with all training conducted at terrestrial altitude (600 m). In addition to normal training, all participants completed four running interval-based training sessions (6 × 1000 m at $v\dot{V}O_{2max}$) on separate days (two in normoxic conditions and two in hypoxic conditions) during the week preceding the 14 d of altitude exposure, and at 11 d and 14 d of hypoxic exposure. The normoxic sessions were conducted on 400 m synthetic running track (600 m terrestrial altitude), whereas hypoxic sessions were conducted at ~3000 m simulated altitude on a motorised

treadmill. Each interval session commenced with a 15 min warm-up, comprised of self-paced, continuous low intensity running, followed by 2 min dynamic stretching.

3.3.4 Sample collection

Blood sampling was performed at three time points (baseline, 3 d and 14 d) during the 14 d altitude camp. Participants provided 2 mL of blood from the antecubital vein immediately after 10 min of supine rest to normalise plasma volume shifts [115] into 1 × 2 mL lithium heparin vacutainer tubes. The tube was immediately centrifuged 2200 × *g* for 10 min and plasma was collected into 500 µL aliquots and stored -80 °C until metabolite isolation.

3.3.5 Sample preparation for metabolomics analyses

Metabolites were isolated from plasma samples using the Bligh and Dyer method [278] in randomised batches of 24. In brief, samples were thawed on ice and a 200 µL aliquot was combined at a ratio of 3:1:1 (v/v) of MS grade methanol (MeOH)/ chloroform ($CHCL_3$)/ plasma, followed by two volumes of analytical grade water. To measure extraction efficiency and monitor mass spectrometry performance, the internal standard trans-cinnamic acid-β,2,3,4,5,6-d_6 (Sigma Aldrich St Louis, MO, USA) was added to the MeOH before extraction. Samples were mixed by vortex at each solvent addition for approximately 15 s and shaken for 10 min at 4 ˚C and 1400 rpm in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany). The bi-phasic extract was then centrifuged for 10 min at 4˚C at 16.100 × *g*. The polar phase of the metabolite extract was transferred to micro-centrifuge tubes and the MeOH was removed using a rotary vacuum concentrator. The remaining aqueous extracts were snap frozen using liquid N_2, lyophilised to dryness, and stored at -80 ˚C until analysis. Prior to instrumental analysis, the dried extracts were reconstituted in 50 µL of 0.1% formic acid (in MS-grade water) and maintained at 10 ˚C in the autosampler. Prior to data acquisition, the analytical sequence was randomised by participant, and then time point. To monitor analytical drift and assess precision, quality control (QC) samples were injected after every 5th sample [247]. QC samples were prepared by combining 20 µL of each plasma sample into a single pool; 50 µL aliquots were then dispensed and as described for the study samples.

3.3.6 Liquid chromatography – mass spectrometry analysis

Samples were analysed using a Waters ACQUITY ultra performance liquid chromatograph UPLC (Waters Corp, Milford, MA) system coupled to a SCIEX TripleTOF 5600 mass spectrometer (SCIEX, Framingham, MA). Chromatographic separation was performed using the Waters Acquity BEH C_{18} column (2.1 × 100 mm, 1.7 µm particle size) (Waters Corp, Milford, MA). The elution gradient using 0.1% formic acid (in MS-grade water; Solvent A) and 0.1% formic acid (in MS-grade acetonitrile Solvent B) was as follows: isocratic step at 1% B for 1 min, 1 to 99.5% B in 36 min, maintained at 99.5% B for 2 min, returned to initial conditions for

1 min, and then equilibration at initial conditions for 5 min. The flow rate was 0.3 mL min^{-1}, injection volume was 10 µL, and the column oven was maintained at 35˚C.

Full scan mass spectrometry data in high resolution were acquired in both electrospray ionisation positive and negative (ESI+/-) using a mass range of 50 – 1000 *m*/*z* according to the following parameters: nebulizer gas (N_2) 45 psi, heater gas (N_2) 50 psi, curtain gas 30 psi and ion source temperature of 550˚C. An IonSpray voltage of 5500 V was used for positive-ion acquisition and –4500 V was used for negative-ion acquisition. MS/MS spectra were collected on all acquired samples to aid in metabolite identification. Independent data acquisition was used on the TripleTOF 5600 to obtain MS/MS spectra for the 5 most abundant precursor ions following each survey scan. A sweeping collision energy setting of 35 ± 15eV was applied in the collision cell using nitrogen as the collision gas. An exact mass calibration was conducted automatically before each batch analysis and at every 6^{th} sample thereafter. The instrument was calibrated before the analysis (calibration error less than 3 ppm) using 0.5 mM sodium formate for both positive and negative ionisation. The TripleTOF 5600 mass spectrometer used Analyst control software v1.6 for data acquisition.

3.3.7 Data pre-processing

Data from each MS ionisation mode were grouped and pre-processed separately. Raw MS files were converted to universal mzXML format using MSconvert [279], then processed using XCMS [280] in R (R 3.1.1; http://cran.r-project.org/). Peak detection and alignment were completed using the following parameters: method = 'centWave', ppm = 10, peak width = 5 – 20, snthresh = 6, mzdiff = 0.01, retention time correction method = 'obiwarp', and *mz*diff = 0.01. Experimental drift was corrected using the Quality Control-Robust Spline Correction (QC-RSC) algorithm [281]. Relative standard deviations were calculated for the pooled QC acquisition data (RSD_{QC}) and the sample acquisition data (RSD_{Sample}); and the ratio of RSD_{sample} to RSD_{QC} was also calculated. Features with > 25% RSD_{QC} or RSD ratio < 2 were considered not sufficiently reproducible and removed prior to statistical analysis.

To suppress the mathematical confounding effect of highly collinear data (and to roughly group chemically similar features, such as adducts and isotopes) metabolite features were combined into a single peak using a simple clustering algorithm. First, a Pearson's correlation matrix was calculated, mapping the pair-wise correlation between each metabolite feature. Second, a retention time difference matrix was calculated, mapping the pair-wise difference in retention time between each metabolite feature. Features that had a pair-wise correlation > 0.8 and pair-wise retention time difference < ± one-second were grouped into a single metabolite "cluster". For each cluster, the feature with the largest peak area was then used for quantification. Orphan features were considered as clusters with N=1.

3.3.8 Metabolite identification

Metabolite identifications were established prior to statistical analysis by matching against online spectral libraries (mzCloud, Metlin, HMDB and Massbank). A level-2 putative match was reported when only the MS1 scan and MS/MS spectra matched that of an online spectral library, but the identification was not confirmed via an in-house authentic standard. This process corresponds with the minimum reporting standards for chemical analysis proposed by the Metabolomics Standards Initiative [282]. Identified metabolites are indicated in the statistical table (level 2), including information of most probable identity and *m/z*.

3.3.9 Statistical analysis

The metabolite cluster data from both positive and negative ionisation modes were combined into a single data matrix. Missing values were imputed using the k-nearest-neighbour methodology (k = 3) [283]. Data were log transformed, both to stabilise variance and to approximate the multivariate normal distribution needed for parametric univariate and multivariate statistical modelling.

Before any formal models were tested, principal components analysis (PCA) was performed on the complete data set, with scores plot labelled by QC and Subject. Here the aim is to assess the natural multivariate variance of the data. If the experiment has been reproducibly performed, it is expected for the QC samples to tightly cluster, such that the QC variance is less than the total sample (biological) variance. This is also an opportunity to identify and remove sample outliers resulting from either poor sample preparation or instrument error.

Metabolites were separated into identified and unidentified data sets. For each metabolite in turn, the null hypothesis that there were no differences in population means across the three consecutive time-points was tested using repeated measures analysis of variance (RM-ANOVA). The method described by Storey et al. (2003) was used to control for the probability of false discovery, which is unavoidably inflated through multiple parallel statistical comparisons [284]. A false discovery rate (FDR) of 0.1 was considered appropriate as a first-pass screen to avoid false positive biomarkers, whilst at the same time avoiding false negative results. For the identified metabolites, results were presented in a table of F-scores, p-values, FDR probabilities and mean fold differences (± 95% confidence interval) for 3 d/baseline and 14 d/baseline. Where appropriate, univariate data was also presented as plots of estimated marginal means relative to the factor *Time.*

The identified ion features were then combined into a single multivariate discriminant model using Principal Component projection followed by Canonical Variate Analysis (PC-CVA) [285]. PC-CVA was performed to visualise the multivariate covariance in the data and uncover multivariate latent structure therein. Often univariately weak, but correlated, variables can

become significant when combined into a multifactorial biomarker signature. The number of PCs to be projected into CVA space was determined by identifying the inflection point in the PCA scree plot. Bootstrap resampling/remodelling was used (n = 500) to determine which metabolites significantly contributed to the optimal model ($p \leq 0.05$).

3.3.10 Data mining of unidentified metabolite features

To determine the strength and the direction of the linear relationship between all identified and unidentified significant metabolite features (pFDR ≤ 0.1), undirected network analysis was performed in the form of a spring-embedded plot [254]. First, a Pearson's correlation matrix was calculated, mapping the pair-wise correlation between each significant metabolite feature. The results of the correlation matrix were graphically presented as a network of "*nodes"* linked by "*edges"*. Each node represents a metabolite, such that the size of the node is proportional to the significance of the metabolite (the larger the node, the lower the p-value). Edges represent a spring constant that is proportional to the correlation coefficient between two metabolites. Edges were only included if the correlation coefficient was positive and significant at a critical p-value of 0.001. Once the network was constructed, it was allowed to "relax"- the connected spring-edges compete against each other to pull nodes in a given direction based on the spring constant (i.e. the higher the correlation, the stiffer the spring, and higher the clustering power). The resulting spring embedded plot was viewed as a multivariate cluster analysis, with highly correlated metabolites clustering closer together. To aid interpretation of the network, non-significant but identified metabolites were included in the spring plot (labeled in grey), based on the premise that metabolites performing a similar function, or belonging to a similar class, will cluster. Networks were coded using the graph visualization software Graphviz (www.graphviz.org) using the 'neato' virtual physics model. All statistical analysis was performed using Matlab scripting language, vR2017a (Mathworks, Natick, MA, USA).

3.4 Results

3.4.1 Altitude exposure

Over 14 d participants accumulated 196.2 ± 25.6 h of normobaric hypoxic exposure.

3.4.2 Metabolomics data

After data cleaning and similarity clustering, the LC-HRMS data resulted in the reproducible detection of 1105 metabolite features in positive ionisation mode and 1896 metabolite features in negative ionisation mode. Of the reproducible features, 36 were identified and confirmed metabolites, from classes including amino acids, tryptophan metabolism, glycolysis, purine metabolism, fatty acids and haem metabolites (**Table 3-1**).

Principal components analysis (PCA) showed (**Fig. 3-1**) that the QC samples clustered, and that the QC-variance was much smaller than the sample-variance. Additionally, it can be observed that across the total measured metabolome the between-subject variance was much greater than within-subject variance. There were no sample outliers detected.

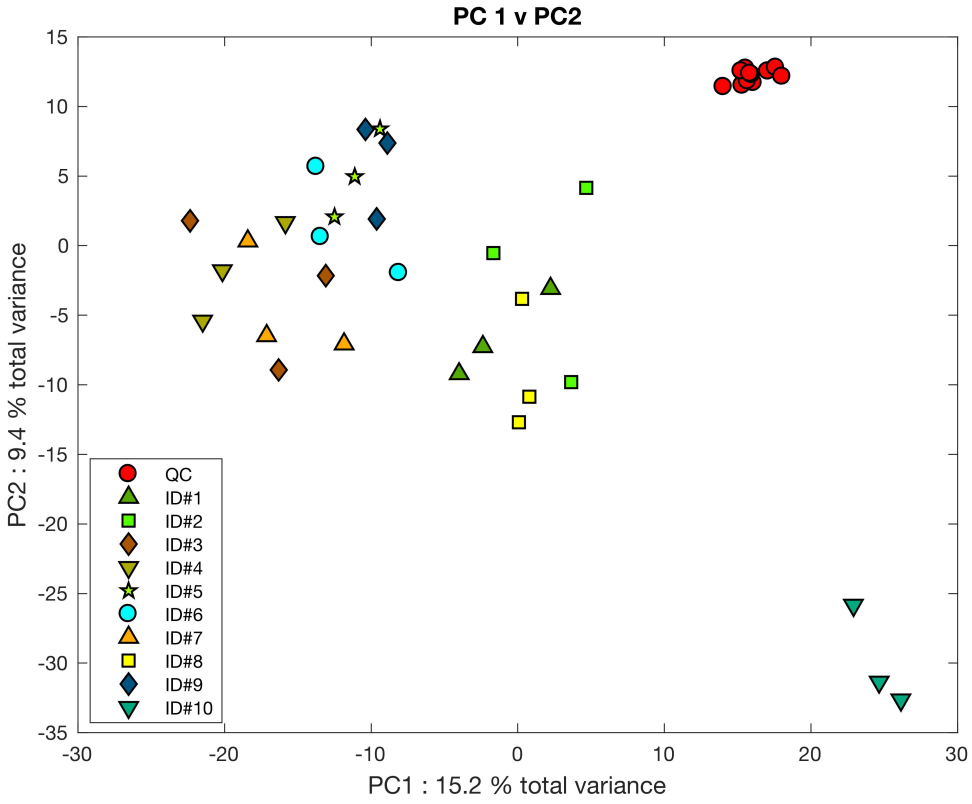

Figure 3-1 Principal components analysis (PCA) scores plot showing all experimental samples of the 3001 reproducible peaks. The quality control (QC) samples (red circles) formed from pooling small quantities from each sample and tightly cluster in the plot.

Table 3-1 Putatively annotated metabolic features during ~3000 m altitude exposure. The table consists of i) the metabolite name, ii) measure m/z, iii) the ANOVA F score, iv) p-value, v) corrected p-value, (vi) & (vii) mean fold change with 95% confidence intervals, & (viii) & (ix) canonical variate scores.

					Day3/1	Day14/1	Canonical variate analysis	
Putative Metabolite	**M+H**	**F (2,18)**	**p-value**	**pFDR**	**Mean fold (95% CI)**	**Mean fold (95% CI)**	**CV1 (95%CI)**	**CV2 (95% CI)**
Bilirubin	585.2706	10.80	0.001	0.04	1.71 (1.36 − 2.16)***	1.25 (-1.01 − 1.57)	-0.26 (-0.46 − -0.06) *	0.1 (-0.08 − 0.28)
Adenosine	268.1045	8.87	0.001	0.04	-2.55 (-4.55 − -1.43)**	-2.32 (-3.92 − -1.37)**	0.13 (-0.13 − 0.4)	-0.18 (-0.34 − -0.03) *
Glycocholic acid	464.2996	7.63	0.01	0.06	-3.34 (-7.56 − -1.48)**	-1.52 (-2.86 − 1.23)	0.24 (0.09 − 0.38) *	-0.04 (-0.24 − 0.16)
Glycoursodeoxycholic acid	448.3049	6.67	0.01	0.06	-3.15 (-6.67 − -1.48)**	-2.15 (-4.16 − -1.12) *	0.21 (0.05 − 0.37) *	-0.06 (-0.24 − 0.12)
Proline	116.0716	6.95	0.01	0.07	-1.34 (-1.68 − -1.06) *	1.03 (-1.13 − 1.19)	0.23 (0 − 0.45) *	0.1 (-0.18 − 0.39)
Tiglylcarnitine	244.1547	5.59	0.01	0.08	1.22 (1.01 − 1.48) *	1.27 (1.09 − 1.48)**	-0.05 (-0.36 − 0.26)	0.17 (-0.06 − 0.39)
Tyrosine	180.0662	5.99	0.02	0.08	-1.49 (-1.93 − -1.16)**	-1.04 (-1.33 − 1.22)	0.22 (0.06 − 0.38) *	-0.03 (-0.24 − 0.18)
Lactic Acid	89.0251	4.93	0.02	0.08	-1.38 (-1.78 − -1.07) *	-1.07 (-1.34 − 1.18)	0.22 (0.03 − 0.42) *	0.03 (-0.19 − 0.25)
Cortisol	363.2173	3.83	0.05	0.17	1.36 (1.02 – 1.81) *	1.03 (-1.22 − 1.28)	-0.2 (-0.42 − 0.02)	-0.01 (-0.22 − 0.19)
Hypoxanthine	137.0464	3.32	0.06	0.18	1.36 (1.01 − 1.84) *	1.12 (-1.16 − 1.47)	-0.18 (-0.41 − 0.05)	0.11 (-0.12 − 0.33)
Methionine	150.0587	3.54	0.06	0.18	-1.29 (-1.74 − 1.05)	1.02 (-1.19 − 1.25)	0.19 (-0.03 − 0.41)	0.1 (-0.12 − 0.31)
2-Octenoylcarnitine	286.2016	3.26	0.07	0.18	1.95 (1.2 − 3.16) *	1.27 (-1.52 − 2.43)	-0.18 (-0.33 − -0.04) *	0.04 (-0.19 − 0.26)
Dodecanedioc acid	229.1438	3.66	0.08	0.19	-2.08 (-5.36 − 1.24)	1.01 (-1.96 − 2)	0.22 (0.01 − 0.42) *	-0.01 (-0.23 − 0.21)
Linoleic acid	281.2480	2.49	0.11	0.26	1.72 (-1.03 − 3.06)	1.24 (-1.35 − 2.07)	-0.08 (-0.3 − 0.13)	0.09 (-0.12 − 0.3)
Phenylalanine	166.0867	2.26	0.14	0.30	-1.32 (-1.83 − 1.06)	-1.16 (-1.55 − 1.15)	0.1 (-0.09 − 0.28)	-0.01 (-0.23 − 0.2)
Tetradecanedioc acid	520.3400	2.28	0.16	0.31	-1.21 (-1.55 − 1.06)	1.1 (-1.15 − 1.4)	0.19 (-0.04 − 0.42)	0.08 (-0.18 − 0.35)
Uric acid	335.0480	1.81	0.19	0.36	1.02 (-1.06 − 1.1)	-1.05 (-1.14 − 1.03)	-0.12 (-0.35 − 0.1)	-0.09 (-0.27 − 0.09)

N-Phenylacetylglutamine	265.1185	1.75	0.21	0.37	1.28 (-1.11 − 1.82)	1.08 (-1.3 − 1.52)	-0.11 (-0.35 − 0.13)	0.02 (-0.22 − 0.26)
Pseudouridine	243.0613	1.64	0.22	0.37	1.1 (-1.05 − 1.28)	1.01 (-1.13 − 1.15)	-0.14 (-0.35 − 0.07)	-0.07 (-0.26 − 0.12)
N-Acetyl-L-Carnosine	269.1245	1.39	0.28	0.44	1.19 (-1.04 − 1.47)	1.08 (-1.17 − 1.37)	-0.12 (-0.35 − 0.11)	0.08 (-0.15 − 0.3)
Tryptophan	203.0822	1.34	0.29	0.44	-1.11 (-1.24 − 1.01)	-1.06 (-1.23 − 1.09)	0.13 (-0.03 − 0.29)	-0.06 (-0.2 − 0.09)
5-Hydroxytryptophan	219.0768	1.25	0.30	0.44	1.15 (-1.04 − 1.37)	-1.02 (-1.39 − 1.33)	-0.17 (-0.41 − 0.06)	-0.09 (-0.36 − 0.17)
Ocatanedioic acid	173.0815	1.21	0.32	0.44	-1.28 (-1.99 − 1.21)	-1.14 (-1.62 − 1.25)	-0.01 (-0.22 − 0.19)	0.02 (-0.19 − 0.23)
Citric Acid	191.0194	0.74	0.44	0.58	1.01 (-1.23 − 1.25)	-1.2 (-1.99 − 1.39)	0.01 (-0.23 − 0.24)	-0.16 (-0.37 − 0.06)
N-N'-Diphenylguanidine	212.1185	0.69	0.46	0.59	1.08 (-1.98 − 2.3)	-1.85 (-10.63 − 3.11)	0 (-0.18 − 0.19)	-0.02 (-0.22 − 0.19)
5-methoxyindole acetic acid	206.0815	0.71	0.49	0.59	-1.1 (-1.27 − 1.05)	-1.03 (-1.26 − 1.19)	0.04 (-0.15 − 0.23)	0 (-0.24 − 0.24)
cis-Aconitic acid	175.0242	0.60	0.50	0.59	1.34 (-1.66 − 2.97)	1.29 (-1.61 − 2.7)	-0.02 (-0.23 − 0.18)	-0.02 (-0.19 − 0.14)
Hexadecanedioic acid	285.2057	0.55	0.54	0.62	-1.09 (-1.64 − 1.37)	1.13 (-1.56 − 1.99)	0.16 (-0.09 − 0.41)	0.06 (-0.16 − 0.28)
Succinic Acid	117.0193	0.42	0.63	0.69	-1.08 (-1.25 − 1.08)	-1.03 (-1.22 − 1.14)	-0.05 (-0.31 − 0.2)	-0.13 (-0.42 − 0.16)
Benzyl cinnamate	239.1069	0.39	0.64	0.69	1.41 (-1.68 − 3.33)	1.53 (-2.49 − 5.83)	0.01 (-0.24 − 0.26)	0.15 (-0.12 − 0.41)
kynurenine	209.0927	0.32	0.69	0.71	1.02 (-1.24 − 1.28)	-1.06 (-1.23 − 1.1)	-0.14 (-0.33 − 0.06)	0.07 (-0.15 − 0.3)
Indole-3-acetic acid	176.0711	0.01	0.98	0.98	1.02 (-1.39 − 1.45)	1.01 (-1.24 − 1.28)	0.03 (-0.18 − 0.24)	0.06 (-0.17 − 0.28)

Note: Significant metabolite response defined as *** $p \leq 0.001$. ** $p \leq 0.01$ and * $p \leq 0.05$. Data are presented as the fold-change of the median (95% CI). Median fold-change and CI were estimated using 500 iterations of bootstrap resampling.

After RM-ANOVA using a critical p-value of 0.03 (pFDR = 0.1), 173 metabolites were found to be statistically significant when compared to baseline (70 in ESI (+) mode and 103 in ESI (-) mode). Of the 36 identified metabolites, eight were statistically significant when compared to baseline. Five metabolites showed a significant disruption followed by recovery trajectory (bilirubin increased at 3 d and then returned to normal at 14 d; glycocholic acid, proline, tyrosine, lactic acid decreased at 3 d and then returned to baseline at 14 d). Three metabolites displayed a significant non-recovery trajectory (adenosine and glycoursodeoxycholic acid decreased at 3 d, but then showed only a slight return to baseline at 14 d; tiglylcarnitine increased at 3 d and then continued to increase to 14 d).

Using a four principal components projection, the results of PC-CVA (**Fig. 3-2 & 3-3**) indicate significant multivariate discrimination between group means ($p < 0.05$). CV1 described a non-linear recovery trajectory from baseline, to 3 d, and then back to the initial position at 14 d. Conversely, CV2 described a weaker, but orthogonal, non-recovery trajectory, increasing from baseline to 3 d, with a further increase from 3 d to 14 d. Examining the CVA loadings plots (**Fig. 3-3**) confirms this observation. The eight significant metabolites in CV1 show a clear correlated recovery trajectory, with bilirubin & 2-octenoylcarnitine observing an initial increase in metabolite concentration then recovery, rather than the initial decrease and then recovery observed in glycocholic acid, glycoursodeoxycholic acid, proline, tyrosine, lactic acid and dodecanedioic acid. Only adenosine was significant in CV2, which reflects the already observed significant univariate non-recovery trajectory.

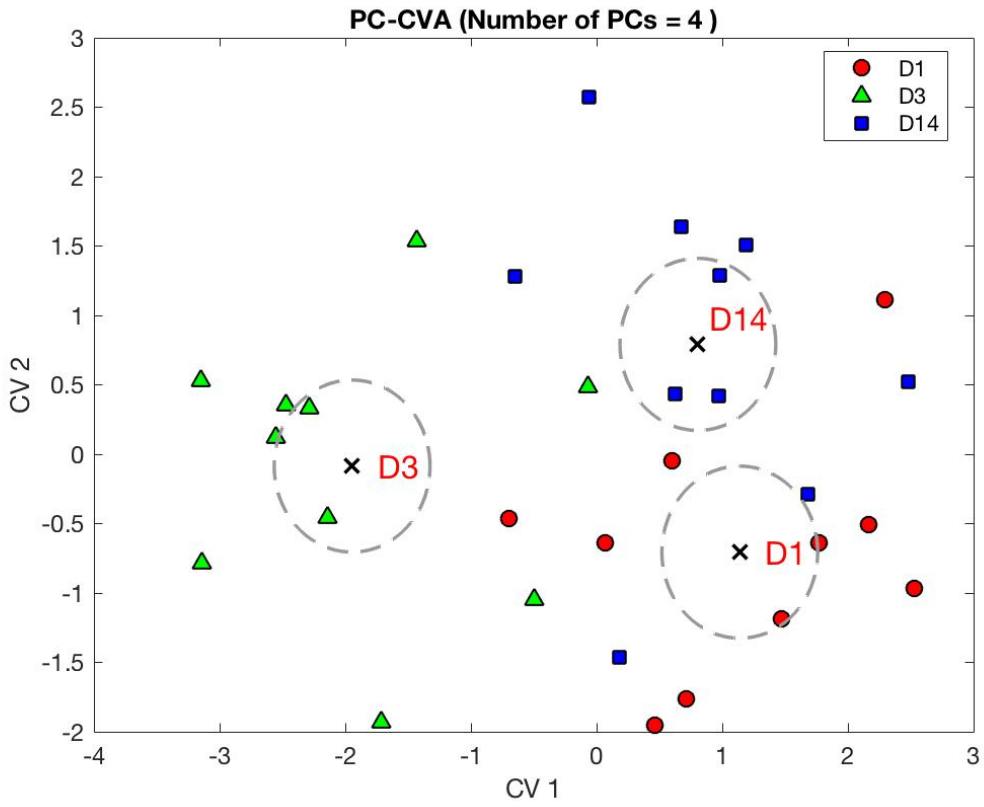

Figure 3-2 Principal Component-Canonical Variate Analysis (PC-CVA) of metabolite levels during the course of hypoxic exposure. Red circles, controls; green triangles, 3 days hypoxic exposure; blue squares, 14 days hypoxic exposure. X, mean of each time point; dashed circles 95% confidence interval of the mean of each time point.

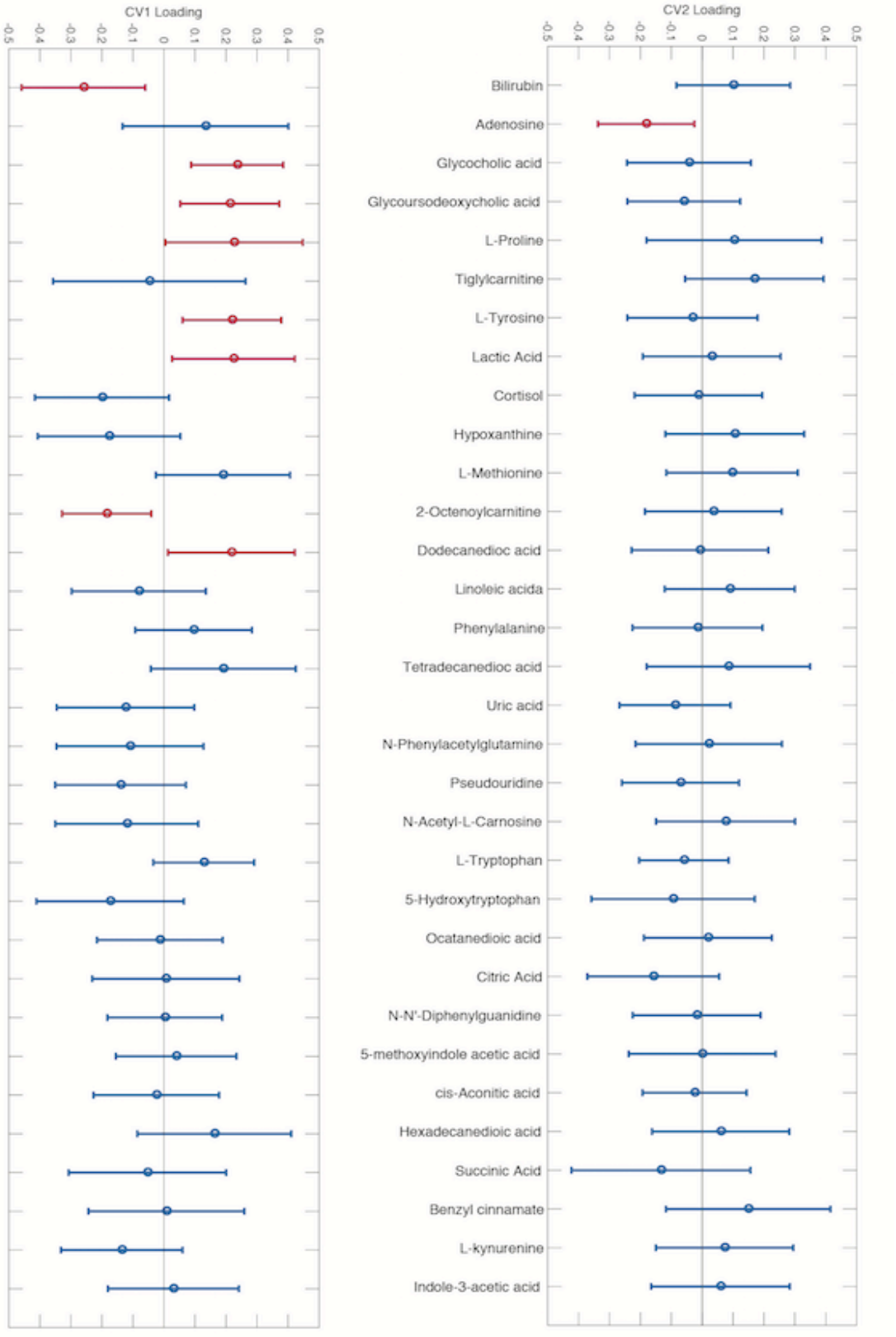

Figure 3-3 Principal Component-Canonical Variate Analysis (PC-CVA) loadings plot of putatively identified metabolites during the course of hypoxic exposure. Metabolites contributing to CV1 (left) and CV2 (right). Red metabolites that significantly ($p < 0.05$) contribute to the separation of each respective CV; blue metabolites that do not contribute significantly to the separation.

The spring embedded correlation plot (**Fig. 3-4**) included unidentified significant metabolite features (pFDR < 0.1) together with all identified metabolites. Three clusters were identified. One small cluster (blue) was composed of metabolites in which metabolite concentration linearly decreased over time and correlated with identified metabolites classes from purine pathways, including adenosine. The second cluster (light blue/green) decreased between baseline and 3 d but then observed clear recovery toward normal metabolite concentration. The third cluster (red – red/green/yellow) increased between baseline and 3 d, then metabolites in the centre of the cluster (dark red) continued to increase at 14 d (this sub-cluster had no associated identified metabolites), whereas the metabolites at the periphery of this cluster showed clear recovery toward normal concentration. This outer cluster includes metabolites associated with steroid metabolism (cortisol), haem breakdown (bilirubin) and purines (hypoxanthine).

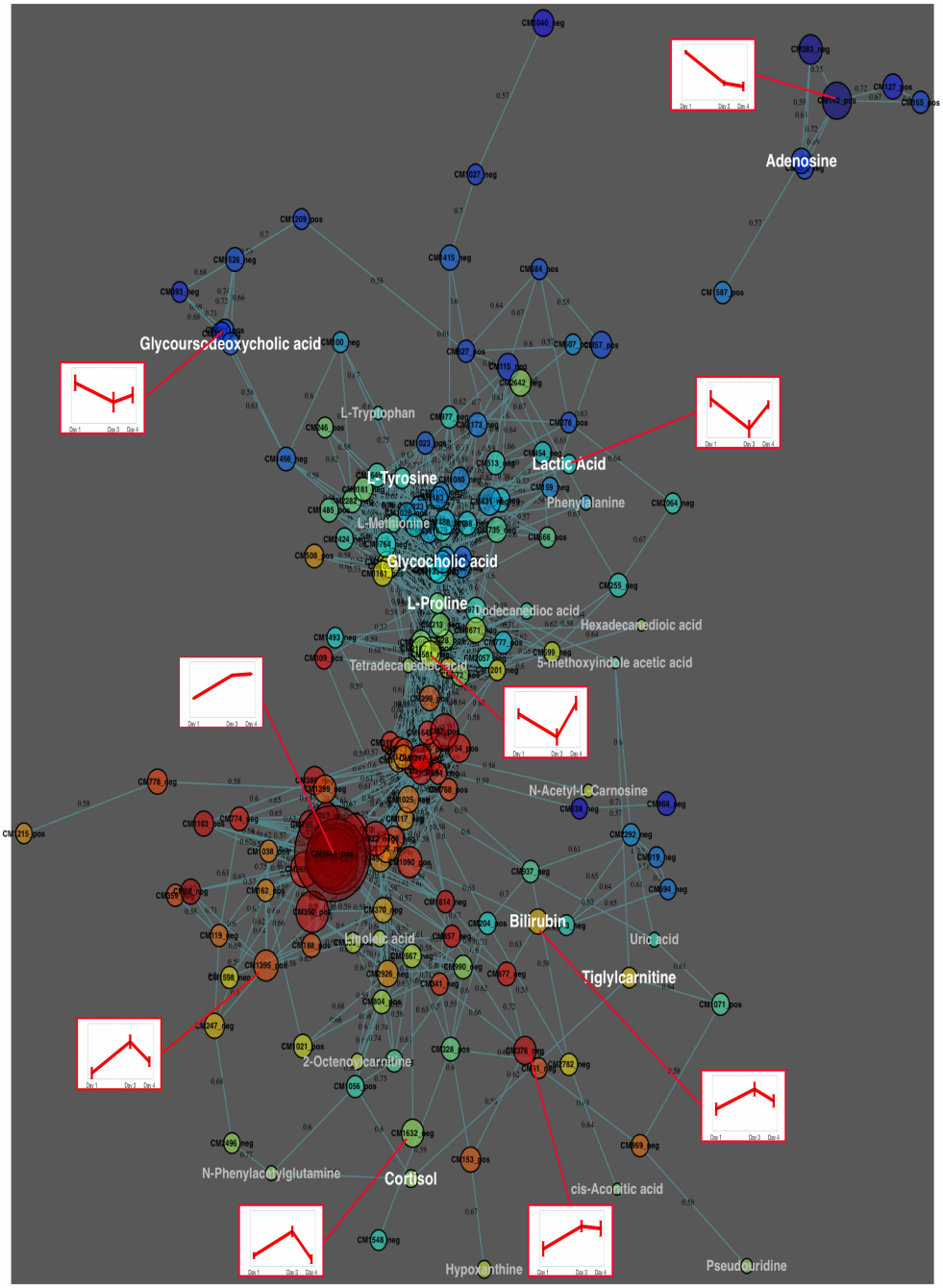

Figure 3-4 Spring embedded correlation plot of the identified and significant peaks following altitude exposure. Metabolites (circles) and the associated correlations (lines/springs). Size of the circle is proportional to the significance of the metabolite (i.e. the larger the circle the more significant the metabolite) and the spring relates the amount of correlation (i.e. the shorter the spring the more correlated the response of the metabolite to its neighbour). The direction of change is indicated by colour, red is increased in concentration and blue is decrease in concentration relative to baseline. Node colour directly maps to the linear correlation coefficient between metabolite concentration and time (red = positive correlation; blue = negative correlation).

3.5 Discussion

This study examined the metabolic changes following 14 d of normobaric hypoxia (~3000 m) in well-trained endurance runners. Using metabolomics and multivariate methods, revealed a metabolic profile associated with acute altitude exposure. There was a significant shift in the plasma metabolite profile following 3 d of acute altitude exposure with partial return towards baseline by 14 d. The metabolites contributing to this change in metabolic profile following altitude exposure followed two separate trajectories.

While several haematological and non-haematological changes are well defined following hypoxic exposure [46, 286], to our knowledge the present study is the first to demonstrate a clear perturbation in an athlete's metabolite profile during 14 d or 196.2 ± 25.6 h of moderate altitude exposure. Univariate statistical analysis revealed that 173 (p-value of 0.03 [pFDR = 0.1]) metabolites were altered at 3 d, 14 d or both. Altered metabolites were observed from distinct metabolite classes: amino acids, tryptophan, glycolytic, heme and nucleotide metabolic pathways. Changes in plasma amino acids were observed to decrease and may reflect metabolic remodelling in order to meet energy requirements. Decreased concentration of several plasma amino acids, such as proline and tyrosine during altitude exposure may reflect utilisation of glucogenic substrates [49, 287]. Horsecroft and Murray [288] recently suggested promotion of amino acids as energy substrates are favoured during high altitude exposure. Interestingly, no evidence of an increased lactate concentration was measured during moderate altitude exposure (fold change -1.38 at 3 d and -1.07 at 14 d), indicating anaerobic metabolism may have been unaffected at the blood sampling time points. These findings support the 'lactate paradox' theory where lactate concentration decreased with increased period of exposure to altitude [184]. Alternatively, increased oxidisation may have occurred to prevent blunting of EPO response during early altitude exposure [289] (**Table 3-1**). Additionally, when compared to baseline, we observed no differences in citrate or succinate, indicating normal TCA cycle activity which is not in agreement with previously published data. For example, Liao et al. (2016) reported increased concentration of blood lactate, succinate and citrate in 60 healthy male volunteers exposed to 5300 m of altitude [49]. It is possible that differences between our findings and those of Liao et al. (2016) are methodological in nature as varying effects on metabolic pathways have been observed between moderate (2000 – 3000 m) and high (3000 – 5500 m) altitudes [269]. It is therefore likely the use of moderate altitude exposure in the current study would likely have promoted different physiological responses when compared with studies completed at higher altitudes.

Canonical Variate Analysis (CVA) revealed that the plasma metabolome was significantly altered during (3 d) and following (14 d) moderate altitude exposure of ~3000 m (**Fig. 3-2**). This finding was expected as altitude exposure places considerable stress on the body

to maintain oxygen driven energetic pathways and redox homeostasis. Importantly, changes in the plasma metabolites were observed at 3 d, with a return to baseline levels by 14 d. The trajectory of the CVA aligns with the acclimatisation response that typically occurs during the first 14 d of altitude exposure [290]. During this period, a process of biochemical and metabolic changes essential for adaptation to lower oxygen environments occur [290]. Although many of the metabolites contributing to alteration in the athlete's metabolome remain unidentified, our findings highlight two distinct adaptation patterns occurring in athletes exposed to moderate altitude. For example, whilst CV1 demonstrates that metabolites initially decreased during the early phase of altitude adaptation, these metabolites appeared to return to baseline levels by 14 d. In comparison, whilst the second trajectory (CV2) initially appeared to either increased or decreased, it did not return to baseline. To this end, further investigation of these metabolites during moderate altitude exposure may help to more accurately describe the complex physiological and metabolic adaptions that occur in athletes during prolonged, moderate altitude exposure, which may in turn provide further insights into the physiological basis of the intra- and inter- individual differences reported in athletes during altitude exposure [276].

Using spring embedded correlation plot (**Fig. 3-4**), identified metabolites that congregate around similar clusters likely share related biological functions and/or metabolic pathways. Accordingly, it is possible to infer that unidentified metabolites that also congregate around clusters of identified metabolites potentially have a similar biological function. We observed tightly clustered metabolites for the identified and significant non-identified metabolites suggesting that these clusters were of similar biochemical relevance. For example, the close clustering of several identified and unidentified amino acids suggests that these metabolites are involved in a similar biological pathway. However, other metabolites originating from the same pathway, whilst detected, were either unable to be identified or not significant, possibly due to the small size (n = 10) within this study. Additionally, the lack of a control group and diet control in the experimental design of this study meant that the biological origin of highly significant features were unknown. To this end, studies tracking larger sample of endurance athletes during sojourns to moderate altitude may provide a more complete description of the complex interaction between hypoxia and the athlete's metabolome. Finally, as this was an observational study it was not possible to analyse athletes' training volume as covariates into our statistical model, thus it is recommended future studies account for these factors when determining the influence of altitude exposure on the blood metabolome.

While preliminary, this study demonstrates the potential for identifying an athlete's unique metabolic signature associated with the typical haematological and biochemical perturbations resulting from moderate altitude exposure. However, the influence of the prolonged hypoxia on the athlete's metabolome remains incomplete, and further research is necessary to determine how different hypoxic training modalities influence the athlete's metabolomics profile.

For example, future studies could include more sampling time points to yield a more complete understanding of the adaptation trajectory of metabolites. To this end, developing less invasive biofluid sampling mediums/matrices such as urine and blood spots, could allow metabolic changes to be more easily quantified during moderate altitude. Furthermore, monitoring an athlete's metabolomic response in the weeks after altitude exposure may permit a better understanding of the physiological factors underlying improved endurance performance upon return to sea level.

3.6 Conclusion

In conclusion, a metabolomic analysis was able to identify that 204 significantly perturbed metabolites contributed to differences in the plasma metabolome during moderate, simulated altitude exposure. It is apparent from the present study that many metabolites remain perturbed beyond 14 d of exposure and further investigation using more targeted methods may provide novel insights into the physiological responses to altitude exposure. Finally, the outcomes of this study demonstrate the potential for metabolomics to provide more detailed information about the physiological adaptations associated with moderate altitude training.

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Conflict of interest: The authors have no conflict of interest to declare.

Chapter 4 Blood removal influences pacing during a 4-minute cycling time trial

Lawler, N.G., Abbiss, C.R., Raman, A., Fairchild, T.F., Maker, G.L., Trengove, R.D., Peiffer, J.J. (2017) Blood removal influences pacing during a 4-minute cycling time trial. *IJSPP*. 2017:1-27 Impact factor: [3.042]

Link: Findings from Chapter 3 indicate acute metabolic adaptions occur in response to low oxygen carrying capacity during moderate altitude. A finding such as a shift in substrate utilisation during acute hypoxia is important. However, the relevance of the shift on both performance is unknown. Given the importance of anaerobic contribution within performance trials the second study sought to explore if the energy system shift could positively impact performance.

4.1 Abstract

Purpose: To examine the influence of manipulating aerobic contribution following whole blood removal on pacing patterns, performance and energy contribution during self-paced middle distance cycling. **Methods:** Seven male cyclists (33 ± 8 y) completed an incremental cycling test followed 20 min later by a 4-min self-paced cycling time trial (4MMP) on six separate occasions over 42 d. The initial two sessions acted as a familiarisation and baseline testing after which 470 mL of blood was removed with the remaining sessions performed 24 h, 7 d, 21 d and 42 d following blood removal. During all 4MMP trials, power output, $\dot{V}O_2$, and aerobic and anaerobic contribution to power were determined. **Results:** 4MMP average power output significantly decreased by 7 ± 6%, 6 ± 8% and 4 ± 6% at 24 h, 7 d and 21 d following blood removal, respectively. Compared with baseline, aerobic contribution during the 4MMP was significantly reduced by 5 ± 4%, 4 ± 5% and 4 ± 10% at 24 h, 7 d and 21 d, respectively. The rate of decline in power output upon commencement of the 4MMP was significantly attenuated and was 76 ± 20%, 72 ± 24% and 75 ± 35% lower than baseline at 24 h, 21 d and 42 d, respectively. **Conclusion:** The results indicate that the removal of 470 mL of blood reduces aerobic energy contribution, alters pacing patterns and decreases performance during self-paced cycling. These findings indicate the importance of aerobic energy distribution during self-paced middle distance events.

Key Words: self-paced, performance, anaerobic capacity, aerobic power

4.2 Introduction

The influence of blood manipulation on exercise performance is complex and likely associated with both haematological and non-haematological alterations that influence aerobic and anaerobic metabolism [174]. For instance, enhancing oxygen delivery through increasing haemoglobin mass (Hb_{mass}) via chronic altitude exposure [27, 173, 291], red blood cell infusion [292, 293], or erythropoietin administration [12] can increase aerobic capacity and exercise performance. Whereas reductions in plasma volume and Hb_{mass}, following the removal of blood [112, 118, 198, 201, 294], the partial blockade of oxygen binding with haemoglobin through carbon monoxide administration [295], and decreasing arterial oxygen content through acute altitude induced hypoxemia [77] can compromise maximal aerobic capacity ($\dot{V}O_{2max}$) and exercise performance. This influence may be especially apparent in middle distance events, which are characterised by a high reliance on aerobic metabolism and where a high level of aerobic fitness is common [43].

Despite the importance of $\dot{V}O_{2max}$ to middle distance events, it is possible that anaerobic energy stores may have a considerable influence on self-selected pacing. Indeed, it has been hypothesised that individuals will manipulate pace throughout an event in order to spare their 'anaerobic energy reserve' for the final 10 – 15% of the effort [43]. Manipulation of diet [67, 296] (i.e. caffeine intake) and motivation [297] augment the distribution of anaerobic energy contribution, resulting in altered pacing and improving performance during middle distance events. Due to the high aerobic demand during middle distance events, it is plausible that reducing aerobic delivery through decreasing blood volume may alter overall pacing through an increased reliance on anaerobic metabolism. However, to date, the influence of altering aerobic energy contribution on the distribution of energetic resources and pacing during middle distance performance is unclear.

The purpose of this study was to determine the influence of voluntary blood donation on pacing and energy resources during self-paced middle distance cycling in trained cyclists. We hypothesised that blood removal would decrease $\dot{V}O_{2max}$ and exercise performance as well as alter the overall pacing profile. We further hypothesised that the changes in haematological measures in the 42 d following the blood removal would result in a gradual recovery in $\dot{V}O_{2max}$ as well as performance and the aerobic contribution during the self-paced cycling task.

4.3 Methods

4.3.1 Participants

Seven trained male cyclists (mean ± SD; age: 33 ± 8 y, height: 1.8 ± 0.1 m, body mass: 72.1 ± 6.9 kg, $\dot{V}O_{2max}$: 60.7 ± 5.5 $mL \cdot kg^{-1} \cdot min^{-1}$, maximal aerobic power: 401 ± 38 W) volunteered to participate in this study. Participants had previous experience performing cycling

time trials and were regarded as trained based on previous classifications [59]. Individuals with a history of hypertension, taking prescribed medications, or deemed as ineligible to donate blood as per the Australian Red Cross blood donation questionnaire were excluded from the study. Participants were asked to maintain regular training commitments and record (hours and distance per week) throughout the duration of the study, and to refrain from heavy exercise in the 24 h period preceding each test and fast (water allowed) for at least 8 h prior to each trial. Participants were informed, verbally and in writing, of the possible risks and benefits associated with the study and signed informed consent was obtained prior to data collection. Ethical approval for this study was obtained from the Human Research Ethics Committee of the participating institution prior to study commencement.

4.3.2 Experimental Design

On separate days, over a period of 50 d, participants made one visit to an Australian Red Cross donation centre and attended six laboratory sessions at the same time of day (± 1 h) in a rested state. During all laboratory visits, participants performed identical exercise consisting of an incremental cycling test followed 20 min later by a maximal 4-min cycling time trial (4MMP). During the initial familiarisation session, participants performed an incremental cycling test and 4MMP habituation trial. This session was followed 7 d later by the baseline testing session. Approximately 24 h after completing baseline testing, participants then attended the Australian Red Cross donation centre for the removal of ~470 mL of whole blood. Following blood donation (24 h, 7 d, 21 d and 42 d) participants repeated the exercise testing session (i.e. incremental cycling test and 4MMP).

In all testing sessions, with the exception of the familiarisation session, a capillary blood sample via finger stick was obtained prior to the start of exercise for the assessment of haemoglobin concentration ([Hb]) and haematocrit (Hct). To avoid the influence of plasma volume shifts,[115] participants remained in a seated upright position for 10 min prior to and during the blood sampling. Haemoglobin concentration was measured using a Hemocue blood photometer (Hemocue® Hb 201, Angelholm, Sweden). Haematocrit was measured using heparinised Micro-Hematocrit capillary tubes centrifuged at 13,500 x *g* for 5 min and assessed using a micro capillary reader.

4.3.2.1 Incremental Cycling Test

Participants completed an incremental cycling test on an electronically braked Velotron cycle ergometer (RacerMate; Seattle, WA, USA) to determine maximal aerobic power (MAP) and $\dot{V}O_{2max}$. The test commenced at 70 W and increased 25 $W \cdot min^{-1}$ until exhaustion. Throughout the test, expired gases were continuously measured using a calibrated metabolic cart (ParvoMedics, TrueOne 2400, Sandy, UT, USA). Measures of oxygen consumption were

obtained as 30 s mean values with ($\dot{V}O_{2max}$) determined as the greatest 30 s mean value recorded during the test. Heart rate was measured using a Polar heart rate monitor (Polar Electro, Kempele, Finland) and power output was measured via Velotron internal software at a frequency of 1 Hz. Immediately following the incremental cycling test, participants were required to complete 5 min of active recovery on the cycle ergometer and then 15 min of passive seated rest prior to the start of the 4MMP.

4.3.2.2 4-Minute Self-Paced Time Trial (4MMP)

Participants performed the 4MMP on the same Velotron cycle ergometer as the incremental cycle test. During the 4MMP, participants were instructed to produce the highest average power output possible over the 4 min duration. As the performance trial was completed after the incremental cycling test, no additional warm-up was provided. All participants started the trial from a standing position using a standardised simulated gear ratio of 52 × 19. During the 4MMP, participants were able to alter their power output by altering their pedalling cadence and gear ratio as required. Only elapsed time was provided to the participants during the trial. Throughout the 4MMP, expired gases, power output and heart rate were measured continuously. Ratings of perceived exertion were measured at 2 min and 4 min using the Borg 10 point scale [298].

4.3.3 Data Processing

Mean power output and cardiorespiratory data ($\dot{V}O_2$, heart rate and respiratory exchange ratio) were calculated for the entire 4MMP cycling trial. In addition, the pacing responses of each trial were assessed using eight 30 s serial splits (e.g. 30 s, 60 s,….., and 240 s). Mean 30 s data were calculated for power output, heart rate, respiratory exchange ratio and $\dot{V}O_2$. To further investigate pacing pattern, a linear regression was fitted to the power output during the first four 30 s splits to determine starting strategy and the rate of decline during the first 2 min of the 4MMP [299].

Metabolic aerobic power was calculated by multiplying oxygen consumption with the oxygen equivalent (as per previous research [300]):

$$\text{Metabolic Power (W)} = \dot{V}O_2\ (L \cdot min^{-1}) \cdot ((4.94\ kJ \cdot L^{-1} \cdot RER + 16.04\ kJ \cdot L^{-1}) / 60)$$

Where $\dot{V}O_2$ is the oxygen uptake in $L \cdot min^{-1}$ and RER is the respiratory exchange ratio. During the 4MMP cycling trials, RER values in excess of 1.0 were adjusted to 1.0 in the calculation of metabolic work per unit of time.

Subsequently, the mean power output and $\dot{V}O_2$ during each split were used to calculate the aerobic contribution (P_{aer}) to power output by multiplying metabolic work by gross efficiency. Gross efficiency was estimated using previous research of participants with characteristics similar

to those in this study [301]. The anaerobic contribution (P_{an}) to power output was calculated by subtracting the P_{aer} from total power output [70].

$$P_{an} = \text{power output} - P_{aer}$$

4.3.4 Statistical Analysis

Weekly training volume, Hct, [Hb], $\dot{V}O_{2max,}$ maximal heart rate, as well as MAP during the incremental exercise test were compared between trials using a one-way repeated measures analysis of variance (ANOVA). Similarly, mean heart rate, rating of perceived exertion and the linear regression fitted to power output during first four splits of each 4MMP cycling trial (i.e., slope) were analysed using a one-way repeated measures ANOVA. Dependent variables during the 4MMP (power output, $\dot{V}O_2$, P_{aer} and P_{an}) were compared between trials and splits using linear mixed modelling with trials (baseline, 24 h, 7 d, 21 d and 42 d) and splits (i.e. 30 s, 60 s) set as fixed factors. Where a significant main effect or interaction was observed, Fisher's least significant difference post-hoc was used to identify where differences occurred. Furthermore, power output was compared between trials by the interaction effect between trial and splits for determination of pacing patterns. Effect sizes (ES) were calculated to determine the magnitude of differences between trials for Hct, [Hb], $\dot{V}O_{2max,}$ MAP from the incremental cycling test and for the mean power output, $\dot{V}O_{2,}$ $P_{an,}$ and P_{aer} measured during the 4MMP at the 95% confidence level. ES estimates of 0.2, 0.5, 0.8, and 1.3 were interpreted as small, moderate, large and very large, respectively [302]. Hedges G correction was applied to account for bias from the small sample size [303]. Statistical tests were conducted using SPSS (Version 21; Chicago, IL), with significance accepted at $p \leq 0.05$. Data are presented as mean ± 95% CI.

4.4 Results

4.4.1 Exercise

Results of the one-way ANOVA revealed no differences in weekly training volume across the 42 d period (6.86 ± 3.55 $h \cdot wk^{-1}$, p = 0 .409).

4.4.2 Haematological Parameters

Results of the one-way repeated measures ANOVA revealed no significant effect for trial on [Hb] (*P* = 0.107; **Table 4-1**). However, moderate ES were calculated for the differences in [Hb] when compared to baseline at 24 h [ES: -0.64 (95% CI = -1.71 to 0.43)], with large ES observed at 7 d [ES: -1.25 (95% CI = -2.40 to -0.11)], which continued to 42 d post donation [ES: 1.26 (95% CI = -2.41 to -0.11)].

A difference was observed for Hct (p = 0.001). Post-hoc analysis revealed that Hct was lower when compared with baseline at 24 h (p = 0.006) and 7 d (p = 0.001) with no differences

observed at 21 d (p = 0.121) or 42 d (p = 0.856). Calculated ES magnitudes for the change from baseline were very large at 24 h [ES -2.00 (95% CI = -3.28 to -0.72)], 7 d [ES -1.74 (95% CI = -2.97 to -0.51)], and large for 21 d [ES -1.04 (95% CI = -2.16 to 0.08)], with no effect observed at 42 d [ES 0.11 (95% CI = -1.16 to 0.94)].

The one-way repeated measures ANOVA revealed a reduction in $\dot{V}O_{2max}$ measured during the incremental cycling test (*P* = 0.003; **Table 4-1**). Post-hoc analysis revealed that when compared to baseline, absolute $\dot{V}O_{2max}$ was lower at 24 h (p = 0.010). No differences were observed at 7 d (p = 0.061), 21 d (p = 0.285) and 42 d (p = 0.959), when compared to baseline. Calculated ES magnitudes for the change from baseline were medium at 24 h [ES -0.70 (95% CI = -1.78 to 0.38)], and small at 7 d [ES -0.33 (95% CI = -1.38 to 0.73)], with trivial effects observed at 21 d [ES -0.19 (95% CI = -1.24 to 0.86)] and 42 d [ES 0.00 (95% CI = -1.05 to 1.05)].

Absolute MAP measured during the incremental cycling test was reduced following blood removal (p = 0.001; **Table 4-1**). Post-hoc analysis revealed MAP when compared to baseline was lower at 24 h (p = 0.001) and 7 d (p = 0.001), however, no differences in MAP were observed at 21 d (p = 0.306) or 42 d (p = 0.150). Calculated ES magnitudes for the change from baseline were medium at 24 h [ES: -0.70 (95% CI = -1.78 to 0.38)], 7 d [ES -0.55 (95% CI = -1.61 to 0.52)], and small at 21 d [ES: -0.21 (95% CI = -1.26 to 0.85)] with a trivial effect observed at 42 d [ES: -0.12 (95% CI = -1.17 to 0.93)].

Table 4-1 Incremental cycling test and haematological measures. Results for baseline and 24 hours, 7 days, 21 days, and 42 days after blood removal, mean (95% CI), N = 7

	Length of time after blood removal				
	Baseline	**24 h**	**7 d**	**21 d**	**42 d**
Maximal aerobic power (W)	401 (372 – 429)	371 (341 – 401)*	378 (351 – 406)*	392 (359 – 424)	396 (365 – 426)
Maximal aerobic power ($W \cdot kg^{-1}$)	5.5 (5.1 – 5.9)	5.2 (4.7 – 5.6)*	5.3 (4.9 – 5.5)*	5.3 (4.9 – 5.7)*	5.5 (5.1 – 5.7)
Oxygen uptake ($L \cdot min^{-1}$)	4.3 (3.9 - 4.6)	4.0 (3.7 - 4.3)*	4.2 (3.8 – 4.5)	4.2 (3.8 – 4.7)	4.3 (3.9 – 4.7)
Heart rate (bpm)	183 (176 - 190)	185 (177 – 192)	178 (168 – 188)	180 (174 – 186)	179 (172 – 186)
Haematocrit (%)	46 (44 - 47)	42 (40 – 43)*	42 (40 – 43)*	42 (42 – 45)	43 (43 – 47)
Haemoglobin ($g \cdot dL$)	151 (146 – 156)	145 (137 – 153)	138 (129 – 146)	137 (126 – 148)	142 (137 – 147)

*Significantly less than baseline ($p \leq 0.05$)

4.4.3 4-Minute Self-Paced Time Trial (4MMP)

When compared to a baseline a main effect of trial was observed for mean power output during the 4MMP ($p = 0.002$; **Table 4-2**). A decrease in mean power output was observed for trial at 24 h ($p = 0.001$), 7 d ($p = 0.001$) and 21 d ($p = 0.028$), however, no differences were observed at 42 d ($p = 0.156$). Calculated ES magnitudes for the change in mean power output were small at 24 h [ES: -0.37 (95% CI = -1.42 to 0.68)], which continued on to 21 d [ES: -0.23 (95% CI = -1.28 – 0.82)] post donation. Likewise, a main effect for trial was observed for $\dot{V}O_2$ measured during the 4MMP ($p = 0.001$; **Table 4-2).** Oxygen consumption was lower 24 h, 7 d and 21 d, ($p = 0.001$) respectively. No differences were observed at 42 d ($p = 0.975$). Calculated ES magnitudes for the change in mean $\dot{V}O_2$ were classified as small at 24 h [ES: -0.43 (95% CI = -1.49 to 0.63)] and 7 d [ES: -0.32 (95% CI = -1.37 to 0.74)].

No main effects or interactions were observed for the trial by split analysis of mean power output (**Fig. 4-1)** or $\dot{V}O_2$ ($p = 0.690$ and $p = 0.100$). The one-way ANOVA for the rate of decline in power output during the initial 2 min of the 4MMP indicated a difference between trials ($p = 0.022$; **Fig. 4-2**). Post-hoc analysis revealed that rate of decline in power output was lower when compared to baseline at 24 h ($p = 0.039$), 21 d ($p = 0.038$) and 42 d ($p = 0.045$), but was not different at 7 d ($p = 0.157$).

No differences in ratings of perceived exertion ($p = 0.457$) or heart rate ($p = 0.072$) were observed in any of the trials when compared to baseline (**Table 4-2**).

4.4.4 Energy Contribution

A main effect for trial was observed for P_{aer} ($p = 0.001$; **Fig. 4-3 a**) with a decrease in P_{aer} observed at 24 h, 7 d and 21 d, ($p = 0.001$), when compared with baseline values. No interactions were observed P_{aer} ($p = 0.100$) between trial and splits. No main effects for trial ($P = 0.161$) or an interaction ($p = 0.736$) were observed for P_{an} (**Fig. 4-3 b**). Calculated ES magnitudes for P_{aer} were classified as small at 24 h [ES: -0.38 (95% CI = -1.44 to 0.67)] through to 21 d [ES: -0.32 (95% CI = -1.38 – 0.73)]. Calculated ES magnitudes mean P_{an} were classified as trivial for all trials.

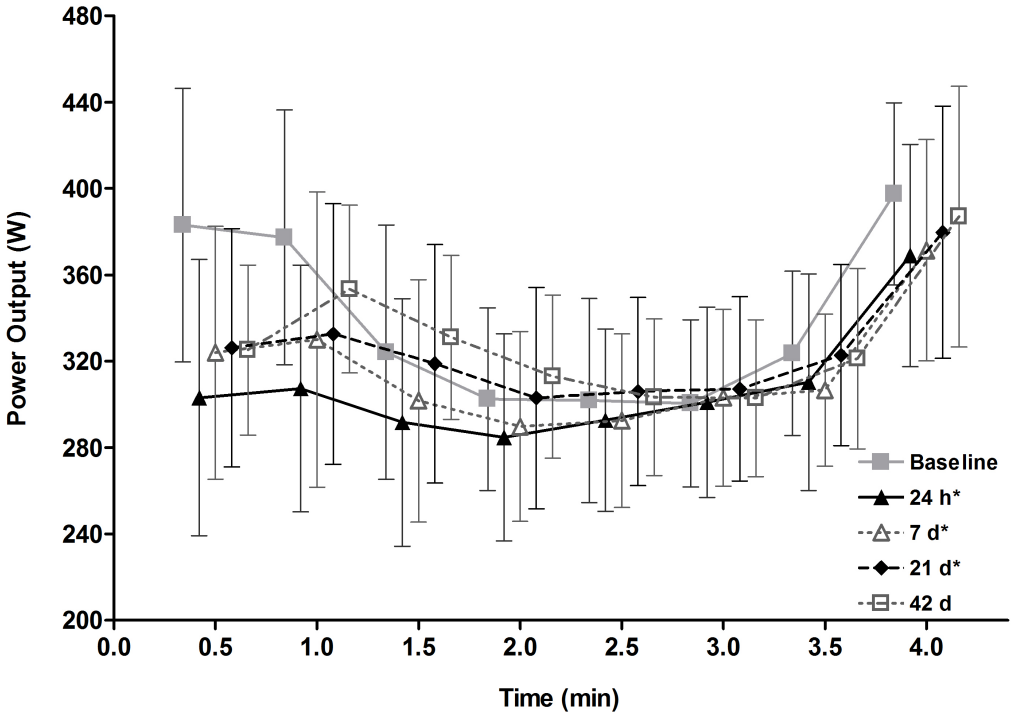

Figure 4-1 Average power output during the 4-minute self-paced cycling time trial (4MMP). Power output (mean ± 95% CI) at baseline and at 24 hours, 7 days, 21 days, and 42 days after blood removal. *p ≤ 0.05; main effect for trial compared with baseline.

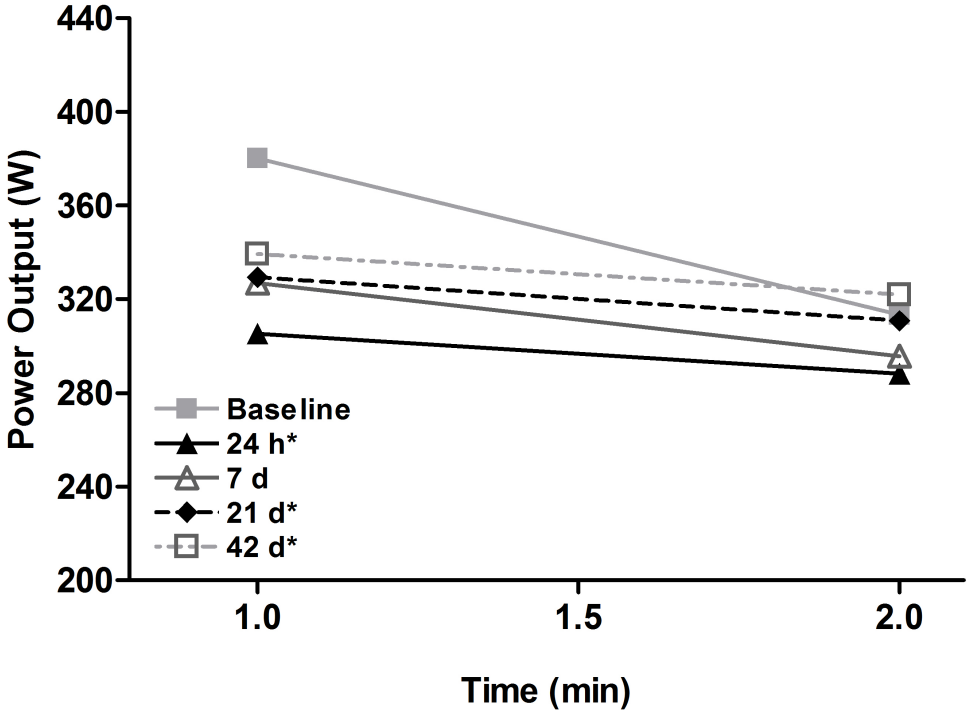

Figure 4-2 Average decline in power output during the first 2 minutes of a 4-minute self-paced time trial (4MMP). Data obtained at baseline, 24 hours, 7 days, 21 days, and 42 days after blood removal. *p ≤ 0.05; significantly different from baseline trial.

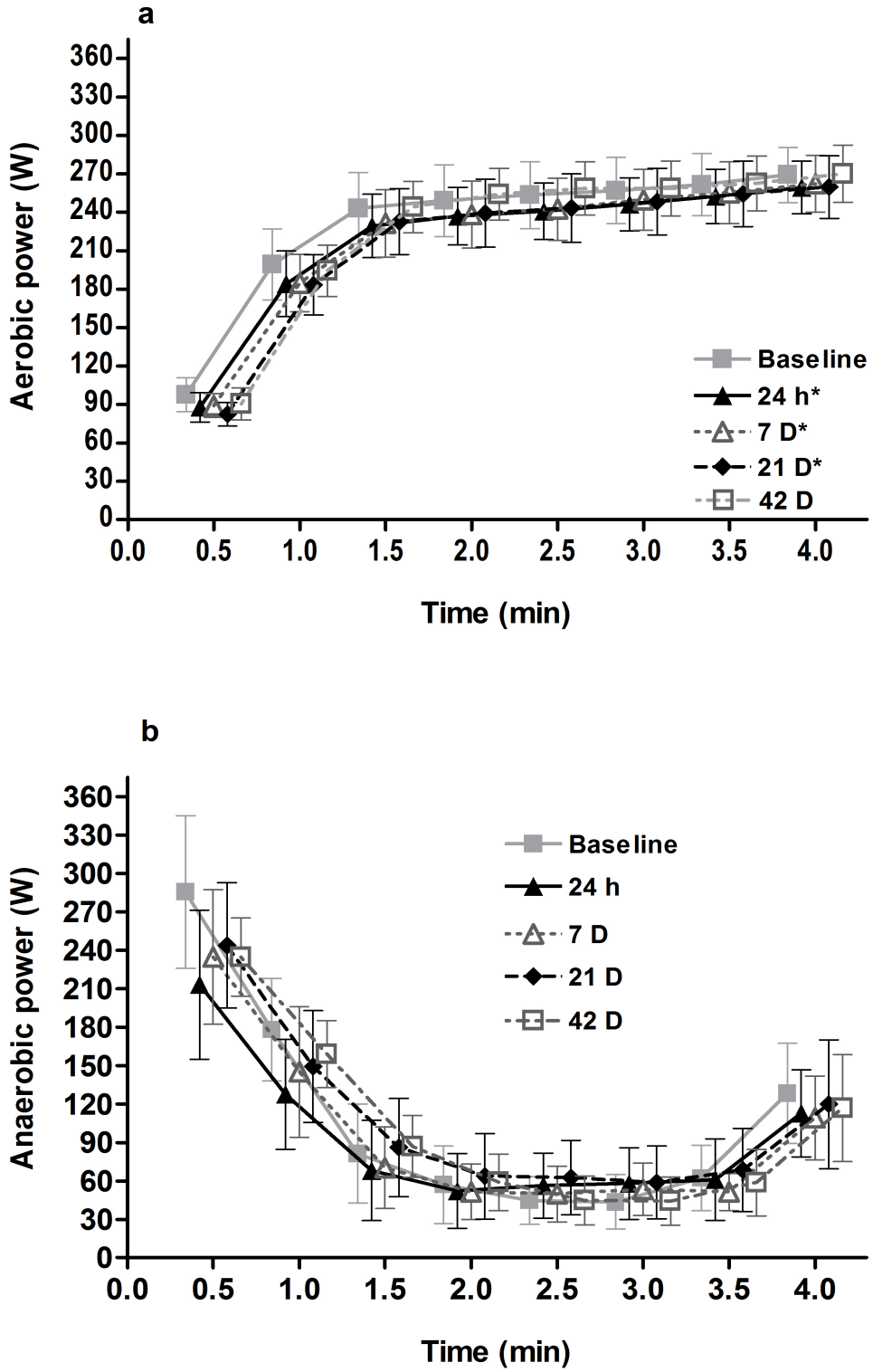

Figure 4-3 Plots of average aerobic (P_{aer}) and anaerobic energy (P_{an}) contributions to power output during the 4-minute self-paced time trial (4MMP). Aerobic (A) and Anaerobic (B) measures obtained at baseline, 24 hours, 7 days, 21 days, and 42 days. *p ≤ 0.05; main effect for trial compared with baseline.

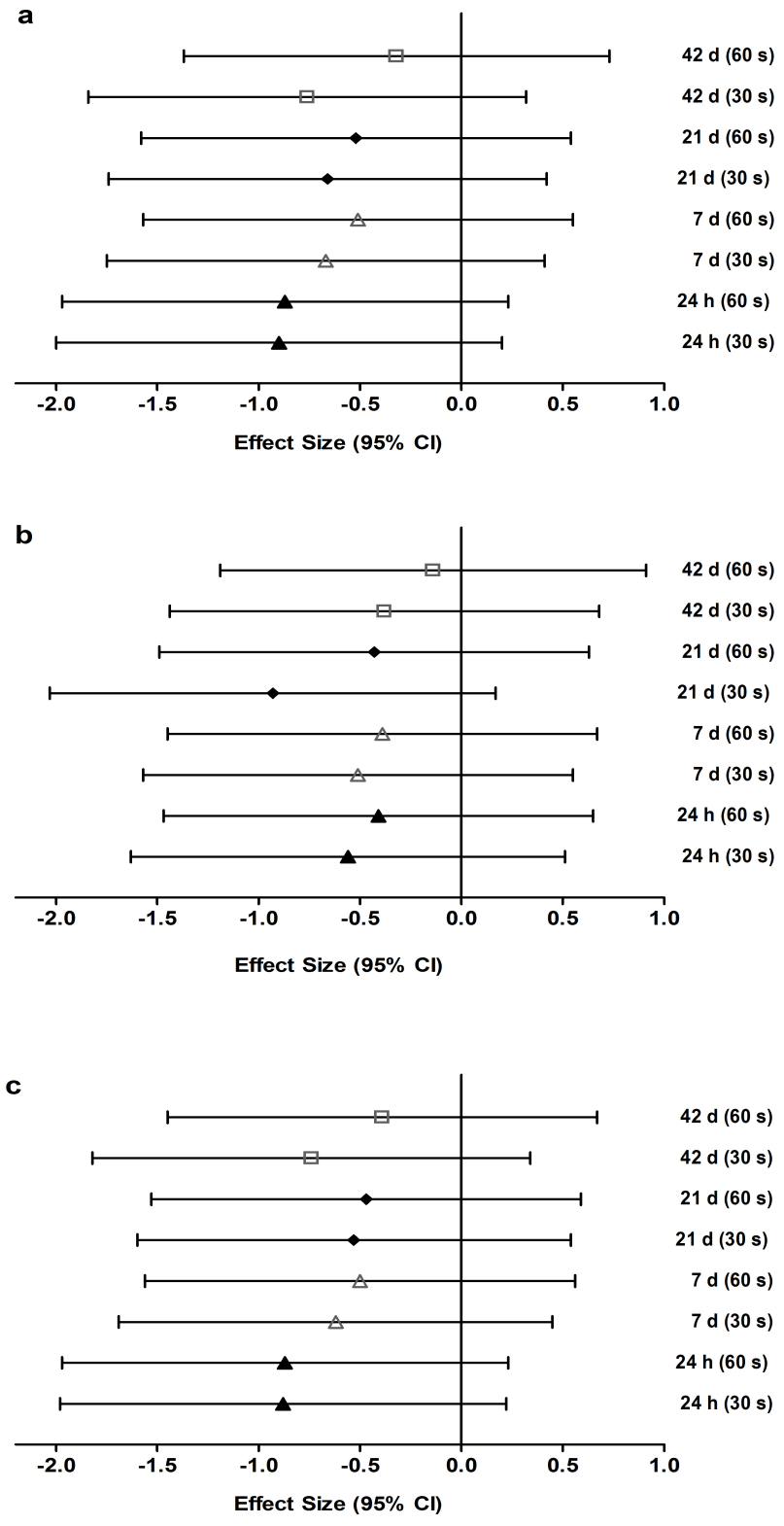

Figure 4-4 Plots of effect-size estimates measured at 30 seconds and 60 seconds of the 4-minute self-paced time trial (4MMP). Differences (± 95% CI) in (a) power output (b) aerobic power, and (c) anaerobic power between 24 hours (filled triangles), 7 days (open triangles), 21 days (diamonds), and 42 days (open squares).

Table 4-2 Overall results for the 4-minute self-paced cycling time trial (4MMP). Results at baseline and 24 hours, 7 days, 21 days, and 42 days after blood removal, mean (95% CI), N = 7

	Length of time after blood removal				
	Baseline	**24 h**	**7 d**	**21 d**	**42 d**
Mean power (W)	339 (297 – 381)	317* (275 – 356)	315 (268 – 361)*	324 (279 – 370)*	330 (293 – 366)
Oxygen uptake ($L \cdot min^{-1}$)	3.6 (3.2 – 3.9)	3.4 (3.1 – 3.6)*	3.4 (3.1 – 3.7)*	3.4 (3.1 – 3.8)*	3.6 (3.3 – 3.9)
Rating of perceived exertion	9.6 (9.0 - 10)	9.9 (9.6 – 10)	9.6 (9.1 – 10)	9.3 (8.8 – 9.8)	9.9 (9.5 – 10)
Heart rate (bpm)	163 (154 – 172)	163 (155 – 172)	162 (153 – 170)	156 (145 – 167)	160 (154 – 167)
Aerobic energy (W)	229 (205 - 252)	217 (199 – 235)*	219 (198 – 241)*	218 (195 – 240)*	229 (210 – 248)
Anaerobic energy (W)	110 (85 – 135)	94 (67 – 120)	96 (69 – 122)	107 (76 – 137)	100 (80 – 121)

*Significantly less than baseline ($p \leq 0.05$).

4.5 Discussion

The purpose of this study was to determine the influence of manipulating aerobic contribution via whole blood removal (470 mL) on pacing patterns, performance and energy contribution during self-paced middle distance cycling. In addition, we examined the influence of blood removal on haematological measures and $\dot{V}O_{2max}$ over the following 42 d. The main findings were: i) $\dot{V}O_{2max}$ returned to baseline values by 7 d despite Hct concentration remaining significantly decreased, ii) the decrease in performance and mean $\dot{V}O_2$ during the 4MMP persisted for 21 d, iii) the rate of decline in power output upon commencement of the 4MMP was less following blood removal, and iv) P_{aer} energy contribution during the 4MMP was reduced for 21 d following blood removal.

Removal of 470 mL of blood resulted in an immediate decrease (24 h) in $\dot{V}O_{2max}$ (-7.5%) measured during the incremental cycling test and is consistent with previous studies [117] reporting -7% [95% CI -11% to -3%] decrease in $\dot{V}O_{2max}$ 24 h – 48 h after removing a similar volume of blood. A decrease in Hb_{mass} (~75 ± 15 g) occurs following the removal of 470 mL of whole blood [197] and supports the notion that reduced oxygen carrying capacity is the primary mechanism responsible for the decline in $\dot{V}O_{2max}$ observed at 24 h. The return of $\dot{V}O_{2max}$ to baseline values at 7 d is likely attributed to plasma volume expansion as a consequence of haemodilution [154] and a resultant increase in stroke volume and cardiac output [304]. In support of this hypothesis, we observed a decrease in Hct at 7 d (**Table 4-1**), and despite no statistical differences, calculated ES estimates for the magnitude of change in [Hb] compared to baseline indicated moderate (ES=-0.64) and large (ES=-1.26) effects at 24 h through to 42 d, respectively.

Power output and $\dot{V}O_2$ during the 4MMP were reduced up to 21 d following blood removal, highlighting the importance of aerobic capacity during middle distance cycling events [60]. Furthermore, despite that the linear mixed modelling revealed no significant differences in power output between trials for any of the 30 s splits (**Fig. 4-4 a**), 'large to moderate' ES estimates were calculated for the differences between baseline compared with all other conditions at 30 s and 60 s. These findings indicate individual pacing patterns were likely influenced by the removal of blood, however, due to the small sample size (n = 7) of our study we were unable to statistically observe these differences. Supporting this interpretation, a greater rate of decline (p = 0.022) in power output over the first 2 min of the 4MMP were observed at baseline, when compared with 24 h, 21 d and 42 d following blood removal (**Fig. 4-2**). Further, these differences were the result of greater power output observed at the start of the baseline trial, when compared with all other trials (**Fig. 4-2**). One possible reason for this change, is a reduction in arterial oxygen content and/or altered $\dot{V}O_2$ kinetics caused by blood removal. Indeed, greater power output upon commencement of middle-distance events is associated with a greater magnitude and/or rate of oxygen consumption [305].

An alternative explanation for the greater decline in power output observed at commencement of the baseline trial was the result of participants adopting a more cautious pacing strategy during subsequent trials. Indeed, the decline in power output at 21 d and 42 d was still lower than baseline, despite markers of aerobic capacity (i.e. $\dot{V}O_{2max}$ and Hct) returning to baseline by 7 d and 21 d, respectively. Within the present study, participants were familiarised with the exercise task and had previous experience performing time trials prior to the baseline 4MMP and as such we hypothesise that changes in pacing at 24 h were not directly caused by a learnt effect. However, it was not possible to blind participants to blood removal or familiarise participants with the trial following blood removal. As such, it is plausible that the cautious pacing at 21 d and 42 d following blood removal were a 'learnt effect' in response to the difficulties encountered during earlier trials (i.e. 24 h). Further research is needed in order better understand the time course by which an intervention may influence subsequent pacing strategies/decisions.

Pacing is dependent on the regulation of energy expenditure [306]. To examine this hypothesis, we measured the contribution of P_{an} and P_{aer} energy resources and relational mechanical power output during the 4MMP. Although the linear mixed modelling revealed no statistical differences in overall P_{an} between trials, we did observe 'large to moderate' ES for differences in P_{an} at the 30 s and 60 s time splits (**Fig. 4-4 c**). Similar differences were observed for P_{aer} (**Fig. 4-4 b**), however, unlike P_{an}, overall P_{aer} contribution throughout each 4MMP significantly decreased following blood removal but returned to baseline by 42 d. Our findings indicate, following blood removal, the differences in P_{aer} likely contributed to the conservative pacing strategy at the commencement of the efforts ultimately resulting in a decrease in performance. These findings are similar to Lima-Silva et al. (2013), Correia-Oliveira et al. (2014), who observed conservative power output distribution following muscle glycogen depletion accompanied by a reduction in P_{aer} [71, 72]. Interestingly, given that total anaerobic energy contribution did not change over the duration of this study, it appears that blood removal does not result in compensatory peripheral adaptations to enhance anaerobic capacity as found following chronic hypoxic exposure [275].

This study provides important insight into the influence of blood donation on performance and pacing during self-paced middle distance events. However, we acknowledge limitations with the study design may have influenced our findings and some interpretation of the data. Following blood removal, performance during the incremental cycling test was reduced, as measured by reduced total time-to-fatigue, $\dot{V}O_{2max}$ (24 h) and MAP (24 h and 7 d; **Table 4-1**). As this test preceded the 4MMP, it is possible that these differences could have influenced the 4MMP results. However, in all instances, we believe participants cycled to fatigue during the incremental cycle test. As such, any differences in total time-to-fatigue, $\dot{V}O_{2max}$ or MAP would have had a minimal influence on the between trial differences in 4MMP performance. The lack of statistical effect observed in this study is likely explained by the small sample size (n = 7) and associated

statistical power. In the absence of additional participants, the inclusion of 95% CI and ES calculations (using hedges G correction) have been provided to highlight findings that, although without statistical relevance, provide insight into the influence of blood removal on pacing and exercise performance. Another potential limitation to this study was the use of an estimated gross efficiency (19.5%) in the calculation of P_{aer} and $P_{an.}$ Although values of gross efficiency were obtained from published data using participants with similar characteristics to this study [301], it is possible the estimated values did not accurately reflect our participants. We would argue, however, that the use of 19.5% efficiency during this study did not impact our findings as the current study was a single group design and inaccuracies in gross efficiency would have minimal influence in the between condition outcomes.

4.6 Practical Applications

Based on the findings in this study athletes and coaches should be aware that aerobic capacity and performance were impaired following the removal of 470 mL. In this study, such impairments to aerobic capacity were evident for up to 42 d following blood removal. Adequate planning should therefore be considered for athletes donating blood prior to competition.

4.7 Conclusions

Results of the present study indicate that the removal of 470 mL of blood reduces $\dot{V}O_2$, performance and overall aerobic energy contribution during a 4MMP. Further, ES indicate variations in pacing patterns during self-paced middle distance events may be associated with changes in the distribution of both, P_{an} and P_{aer} energy resources. Starting strategies appear to be associated with energy distribution as indicated by the decrease in aerobic metabolism following blood removal. Thus, reductions in performance during this study highlight the importance of aerobic energy distribution during middle distance self-paced events.

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Chapter 5 Purine metabolites are associated with the physiological response to standard blood donation

This chapter has been drafted to conform to the author guidelines for the Journal of Science and Medicine in sport.

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Link: Chapter 4 demonstrated the importance of aerobic energy contribution during middle distance cycling events and showed that a substrate shift can occur in low oxygen environments is unable to compensate for a decrease in aerobic capacity. While an acute (14 d) increase in anaerobic energy was observed in Chapter 3, this study (Chapter 5) sought to explore the metabolic changes immediately following and up to 42 d following blood donation.

5.1 Abstract

Purpose: The purpose of this study was to determine the metabolic changes following blood removal using an untargeted metabolomic approach. **Methods:** Thirteen recreationally trained males (age: 29 ± 7 y) attended eight testing seasons (Baseline, 24 h, 7 d, 14 d, 21 d, 28 d, 35 d and 42 d) and, attended one standard blood donation. At each of the time points, plasma samples were collected and stored at -80°C and measures of haemoglobin concentration and haematocrit were taken. At baseline, 24 h, 7 d, 21 d, 42 d, incremental cycling test and cycling time trials were performed. Plasma samples were measured using liquid chromatography high resolution mass spectrometry (LC-HRMS) and analysed using untargeted metabolomics analysis. **Results:** Decreases to maximal power output (7 d), maximal oxygen consumption (24 h), haemoglobin and haematocrit (14 d) as well as average time trial power output (7 d) and oxygen consumption decreased (24 h), $p \leq 0.05$. Metabolomics analysis on the 104 plasma samples identified 152 metabolites of which 40 were deemed significant. Hierarchal Cluster Analysis (HCA) revealed eight metabolite group trajectories. A significant trajectory was identified as purine pathway, which was observed to immediately increase post blood donation followed by a slow transient recovery to baseline levels ~ 42 d. **Conclusion:** Consistent with previous studies, blood removal decreased key haematological parameters for oxygen transport and maximal aerobic capacity. However, changes in $\dot{V}O_2$ and performance during the cycling time trial were not consistent with one another, indicating that fitness and performance are not intrinsically linked. Untargeted metabolomics revealed possible compensatory mechanisms, such as changes in purines, which may occur to enhance availability of oxygen to the muscles.

5.2 Introduction

Blood removal (i.e. blood donation) results in acute decrease in haemoglobin concentration [Hb] leading to a decline in aerobic capacity [112, 197-199]. Indeed, data from a recent meta-analysis indicates that 24 h following a blood donation (~470 mL) [Hb] and maximal aerobic capacity ($\dot{V}O_{2max}$) are reduced some 7% and 3.5%, respectively [117]. The acute (≤ 14 d) impact of blood donation has been examined extensively (see review [117]); with only one study examining the influence of blood donation over the normal red blood cell recovery period (~40 d) (Chapter 4). In a group of trained cyclists, the removal of 470 mL of whole blood resulted in an immediate (24 h) decrease in $\dot{V}O_{2max}$ and peak power output during a graded exercise test; with only $\dot{V}O_{2max}$ demonstrating recovery by 7 d. Additionally, during a self-paced four-min cycling time trial, performance (mean power output) and aerobic contribution (mean $\dot{V}O_2$) were reduced up to 21 d corresponding with a reduction in [Hb] (Chapter 4). From these findings, it can be concluded that changes in [Hb] alone, after blood removal, are not solely responsible for the observed changes in maximal and submaximal performance. As such, further investigation is warranted.

Metabolomics has a history of use in human biological samples [34, 307, 308] and more recently in the area of exercise sciences [35, 246, 260, 263, 309]. This technique provides the ability to comprehensively characterise system level changes in metabolism at a given time point [207]; thus, allowing the description of changes of a metabolic profile to external stimuli. Metabolomics, therefore, represents an ideal analytical technique to understand the impact of blood removal on the human physiology and performance by allowing the visualisation of all of metabolic changes occurring in unison. Indeed, D'Allessandro et al. (2016) have demonstrated the usefulness of this technique during 16 d of high altitude exposure (comparative to blood donation), observing an increase in purine metabolites, adenosine, hypoxanthine and adenosine monophosphate all of which are associated with metabolic adaptions within red blood cells [270].

Blood donation is vitally important to worldwide healthcare [310]. However, individuals who exercise regularly may be reluctant to donate as currently very little data is available for the impact of blood removal on donor physiology and performance beyond 14 d [117]. Furthermore, previous data (Chapter 4) [311] indicates that changes in aerobic capacity and submaximal performance following blood removal are likely modulated by different factors, that as of yet have not been fully identified. Therefore, the primary aim of this study was to investigate the metabolic changes immediately following and up to 42 d post blood removal using an untargeted metabolomics approach. The secondary aim, was to describe the effect of a single whole blood donation (i.e. 470 mL) on maximal aerobic power, $\dot{V}O_{2max,}$ cycling time trial performance and key haematological parameters over 42 d. This study represents an extension to previously published work using a sub-group (n = 7 trained cyclists) of this cohort (Chapter 4).

5.3 Methods

5.3.1 Participants

Thirteen recreationally trained males (mean ± SD; age: 29 ± 7 y, height, 1.8 ± 0.1 m, body mass: 76.8 ± 6.9 kg, $\dot{V}O_{2max}$: 53.0 ± 10.9 $mL \cdot kg^{-1} \cdot min^{-1}$, maximal aerobic power: 363 ± 61 W) were recruited to the study. Individuals were excluded from the study if at enrolment they had previously donated blood ≤3 months, had taken prescribed medications, had history of hypertension, or were found to have [Hb] <130 $g \cdot L^{-1}$. In addition, participants needed to meet requirements to donate blood as per the Australian Red Cross blood donation criteria. One week prior, and during the 42 d, participants were asked to record a daily diet diary (Easy Diet Diary) and to refrain from physical activity and caffeine 24 h prior to experimental trials; all participants self-reported compliance with these measures. Participants were provided with possible risks and benefits of their participation in this study and written consent was obtained prior to data collection. Approval for the investigation was obtained from Murdoch University (Western Australia, AUS) Human Research Ethical Committee.

5.3.2 Experimental design

This study used a quasi-experimental design consisting of a single group with repeated measures. Participants were required to complete a familiarisation session and a baseline testing session, no greater than 7 d apart. Within 24 h of completing the baseline testing, participants attended an Australian Red Cross donation centre for the removal of ~470 mL of whole blood. Additional testing sessions were completed 24 h, 7, 14, 21, 28 35, and 42 d after the blood donation. The testing sessions at 24 h, 7, 21 and 42 d consisted of; (a) venous and capillary blood sampling, (b) an incremental cycling test and (c) 4-min cycling time trial (4MMP) cycling test. On days 14, 28 and 35 participants were only required to provide a blood sample.

5.3.2.1 Incremental cycling test

Participants completed an incremental cycling test on a Velotron cycle ergometer (RacerMate; Seattle, WA, USA) commencing at 70 W and increasing 25 $W \cdot min^{-1}$ until volitional exhaustion. Expired gases were continuously measured using a calibrated metabolic cart (ParvoMedics, TrueOne 2400, UT, USA). Measures of oxygen consumption were calculated as 30 s mean values with ($\dot{V}O_{2max}$) determined as the greatest 30 s mean value recorded. Heart rate was continuously measured using a Polar heart rate monitor (Polar Electro, Kempele, Finland) and power output (W) was measured via Velotron internal software at a frequency of 1Hz. Maximal aerobic power (MAP) was measured as the power output corresponding to the last completed stage plus pro rata value for any incomplete stage.

5.3.2.2 4-Minute self-paced time trial (4MMP)

Twenty min after completing the incremental cycling test, and using the same cycle ergometer, participants completed a 4MMP. During this test, participants were instructed to produce the highest average power output possible during the 4MMP test. No restrictions were placed on cadence or gear ratio throughout the test and only elapsed time was provided to participants as feedback. Power output was continuously collected at a frequency of 1 Hz using Velotron internal software, while heart rate and $\dot{V}O_2$ were measured as 30 s mean values using a metabolic cart.

5.3.2.3 Blood sampling

During all experimental sessions, participants arrived at the laboratory following a nine-hour fasting period (9 ± 1 h). In the laboratory participants were asked to remain seated upright for ten min prior to, and during sampling to avoid plasma volume fluctuations. Blood sampling consisted of capillary sample via finger prick for assessment of [Hb] and (Hct %). Haemoglobin concentration was measured using a Hemocue blood photometer (Hemocue® Hb 201, Angelholm, Sweden). Haematocrit % was measured using heparinised Micro-Hematocrit capillary tubes centrifuged at 13,500 x *g* for 5 min and assessed using a micro capillary reader. In addition, a blood sample (4 mL) was collected from the vein of the antecubital fossa into lithium heparin vacutainer tube (BD Vacutainer) for metabolomics analysis. Whole blood obtained via venepuncture was immediately centrifuged at 1800 × g for 10 min. Resulting plasma was aliquot and stored at -80°C for metabolomics analysis.

5.3.3 Sample preparation for metabolomics analyses

Plasma samples (50 µL) were thawed and extracted using the Bligh and Dyer method [278], such that, metabolite extraction was achieved by adding 3:1:1 (v/v) of ice-cold analytical grade methanol (MeOH)/ chloroform ($CHCL_3$)/ plasma followed by two volumes of analytical grade water. An internal standard, trans-Cinnamic acid-β,2,3,4,5,6-d_6 Sigma Aldrich (St Louis, MO, USA) was dissolved in MeOH and added to each plasma sample. Samples were incubated for ten min in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) at 4 ˚C and 1,400 rpm. Two volumes of analytical grade water were then added and the mixture incubated for ten min at 4 ˚C at [1400 rpm] and then centrifuged ten min at 4˚C at 16100 × *g*. The supernatant was transferred to micro-centrifuge tube and the MeOH was removed using a rotary vacuum. Samples were snap frozen using liquid nitrogen, lyphilised by freeze-drying, and stored at -80 ˚C until analysis. Pooled plasma (quality control) from the 13 participants from all sampled time points (i.e. 104 samples) were prepared and analysed throughout the experimental batches to monitor the analytical variation of the LC-MS system [247]. Before data acquisition, the analytical sequence was randomised by participant, then time point and divided into two batches.

5.3.4 Liquid chromatography – mass spectrometry

Plasma extract samples were reconstituted in 50 µL of 0.1% formic acid in water before LC-HRMS analysis. The samples were analysed using a Waters ACQUITY UPLC (Waters Corp, Milford, MA) system coupled to an electrospray SCIEX TripleTOF 5600 mass spectrometer (SCIEX, Framingham, MA). All samples were analysed separately in positive and negative ion modes. Quality Control (QC) samples were analysed for the first ten injections and then every fifth injection with the final two injections as QC's. Metabolite separation was performed following a 10 µL sample injection onto a Waters Acquity UPLC™ column (BEH C_{18} 2.1 x 100 mm, 1.7 µm; Waters Corp, Milford, MA) with the column temperature set at 35 ˚C. The two solvents applied were solvent A – 0.1% formic acid in water and solvent B – 0.1% formic acid in acetonitrile at a flow rate 0.3µL min^{-1}. Solvent A was held at 99.5% for one min followed by an increase to 99.5% solvent B over 36 min, which was then held at 99.5% for two min. At 39 min, it was changed to 99.5% solvent A and held at 99.5% solvent a to equilibrate for five min. All eluent was collected by the mass spectrometer using full scan using a mass range of 50 – 1000 *m/z*.

Profile mass spectra were acquired in 'high resolution' according to the following parameters: nebulizer gas (N_2) 45 psi, heater gas (N_2) 50 psi, curtain gas 30 psi, and ion source temperature of 550˚C. An IonSpray voltage of 5500 V was used for positive-ion acquisition and –4500 V was used for negative-ion acquisition. Independent data acquisition was performed on representative QC samples, randomly allocated in the acquisition list, to aid in metabolite identification. MS/MS spectra for the five most abundant precursor ions following each survey MS1 scan were collected. A sweeping collision energy setting of 35 ± 15eV was applied in the collision cell using nitrogen as the collision gas. An exact mass calibration was conducted automatically before each batch analysis and at every 6th sample thereafter. The instrument was calibrated before the analysis (calibration error less than 3 ppm) using 0.5 mM sodium formate for positive and negative ionisation, respectively. The TripleTOF 5600 mass spectrometer used Analyst control software version 1.6 for data acquisition.

5.3.5 Data pre-processing

Data from each MS ionisation mode were grouped and pre-processed separately. Raw MS files were converted to universal mzXML format using MSconvert [279], then processed using XCMS [280] in R (R 3.1.1; http://cran.r-project.org/). Peak detection and alignment were completed using the following parameters: method = 'centWave', ppm = 10, peak width = 5 – 20, snthresh = 6, mzdiff = 0.01, retention time correction method = 'obiwarp', and *mz*diff = 0.01. Systematic error in peak-area measurement with respect to injection order, was corrected using the Quality Control-Robust Spline Correction (QC-RSC) algorithm [281]. To suppress the

mathematical confounding effect of highly collinear data all chemical adduct and isotope peaks were removed from the datasets, where possible.

5.3.6 Metabolite identification

Metabolite identifications were established prior to statistical analysis, by matching against online spectral library using ions from the experimental samples. Those with a match of molecular weight (*m/z*), were hand checked against online spectral libraries (mzCloud, Metlin, HMDB and Massbank) for MS spectral match. To increase identification yield, QC samples were run through an Q-Exactive™ high resolution mass spectrometer (Thermo Fisher Scientific, San Jose, USA) using standard protocols and settings. Data was processed using Compound Discoverer™ software (Thermo Scientific). The resulting metabolite identifications were mapped back to the original data set with a mass tolerance of +/- 3ppm. A definitive, level-1, match was reported when a given peaks' retention time, MS1 scan and MS/MS spectra matched that of an authentic standard analysed on the same instrument. A level-2 putative match was reported when only the MS1 scan and MS/MS spectra matched that of an online spectral library, but the identification was not confirmed via an in-house authentic standard. This process corresponds with the minimum reporting standards for chemical analysis proposed by the Metabolomics Standards Initiative [282]. Identified metabolites are indicated in the statistical table, including information of most probable identity, m/z, retention time (RT), elemental formula, and ppm error.

5.3.7 Statistical Analysis

All statistical analyses were performed using Matlab scripting language, version R2017a (Mathworks, Natick, MA, USA). Statistical comparisons between $\dot{V}O_{2max,}$ (MAP) obtained during the incremental cycling test were compared between time points using a repeated measures Analysis of Variance (RM-ANOVA). Similarly, haematological measures Hct %, [Hb], and variables measured during the 4MMP were calculated (mean $\dot{V}O_2$ and power output (W)) and compared between time points using RM-ANOVA. Where significant main effects were observed, Bonferroni's post-hoc were performed. Significance was set as $p \leq 0.05$, and data were presented as mean ± 95% CI.

The metabolite data from both the positive and negative ionisation modes were combined into a single data matrix. Missing values were imputed using the k-nearest-neighbour methodology (k=3) [283]**.** Data were log transformed, both to stabilize variance and to approximate the multivariate normal distribution needed for univariate parametric and multivariate statistical modelling. Before any formal models were tested, the natural multivariate variance of the data was assessed using Principal Components Analysis (PCA) performed on the complete data set, with scores plot labelled by QC and experimental samples.

Metabolites labelled with a putative identification were assessed for significance across the eight consecutive time points using RM-ANOVA. The method described by Storey et al. (2003) was used to control for the probability of false discovery, which is unavoidably inflated through multiple parallel statistical comparisons [284]. For the identified metabolites, results were presented in a table of F-scores, p-values. Where appropriate, univariate data was also presented as plots of estimated marginal means relative to the factor *Time.*

Unsupervised two-way Agglomerative Hierarchical Cluster Analysis (HCA) assessed multivariate similarities between individual's metabolomic profiles. This algorithm used a multivariate Euclidean distance metric and Ward's group linkage. The results were displayed as a circle dendrogram plot with metabolite labels set as black = $p > 0.05$, and red= $p < 0.05$. The cluster dendrograms revealed the lower the linkage in the "tree", the more similar the feature. The emergent clusters have the most similar characteristics and were labelled from A through to H.

5.4 Results

5.4.1 Haematological data

Compared with baseline values, Hct% was lower at 24 h ($p < 0.01$) and 14 d ($p = 0.01$), with no other difference observed between time points. Compared with baseline values, [Hb] was less at 14 d ($p = 0.04$; **Table 5-1**), with no other differences noted between time points.

5.4.2 Incremental Cycling test

$\dot{V}O_{2max}$ decreased following the removal of ~470 mL of whole blood ($p < 0.01$; **Table 5-1**). Compared with baseline, $\dot{V}O_{2max}$ was less at 24 h ($p = 0.02$) with values returning to baseline by 7 d ($p > 0.05$). Absolute (MAP) during the incremental cycling test was reduced following blood removal ($p < 0.01$; **Table 5-1**) with lower values at 24 h ($p < 0.01$) and 7 d ($p = 0.01$) when compared with baseline.

5.4.3 4-Minute Self-Paced Time Trial (4MMP)

Mean power output from the 4MMP decreased from baseline ($p < 0.01$; **Table 5-1**) with lower power output observed at 24 h ($p = 0.02$) and 7 d ($p = 0.03$). A decrease in mean oxygen consumption ($\dot{V}O_2$) measured during the 4MMP was observed ($p = 0.02$), with lower $\dot{V}O_2$ values at 24 h ($p < 0.01$; **Table 5-1**) compared with baseline.

Table 5-1 Summary of haematological measures, incremental cycling test measures and 4-minute self-paced cycling trial (4MMP). Measures obtained baseline and 24 h, 7 d, 21 d, and 42 d after blood removal, (mean [95% CI]), N = 13

	Length of time after blood removal							
	Baseline	**24 h**	**7 d**	**14 d**	**21 d**	**28 d**	**35 d**	**42 d**
Haematocrit (%)	46 [45-47]	42 [40-44]*	43 [41-45]	44 [42-46]*	44 [42-46]	44 [42-46]	45 [43-47]	45 [44-46]
Mean difference [95% CI]		-3.7 [1.0-6.4]	-3.1 [-0.17-6.3]	-2.7 [0.33-5.1]	-1.7 [-1.3-4.6]	-2.0 [-0.67-4.7]	-0.69 [-3.3-4.7]	-0.39 [-3.1-3.8]
Haemoglobin ($g.dL^{-1}$)	154 [148-160]	144 [139-149]	143 [136-150]	141 [135-147]*	141 [134-148]	142 [133-151]	146 [136-156]	147 [140-154]
Mean difference [95% CI]		-10.0 [-2.5 22.5]	-11.0 [-3.9 25.9]	-12.8 [0.04-25.5]	-12.8 [-3.4-29.1]	-11.5 [-3.2-26.3]	-7.8 [-10.8-26.3]	-7.0 [-4.8-18.8]
Incremental cycling test								
Oxygen uptake ($mL.kg.min^{-1}$)	53.07 [47.14-59]	49.96 [44.8-55.12]*	51.55 [46.03-57.07]		52.45 [46.69-58.21]			53.29 [47.82-58.76]
Mean difference [95% CI]		-3.1 [0.36-5.88]	-1.5 [-0.19-3.23]		-0.62 [-1.35-2.60]			-0.22 [-2.39-1.60]
Maximal aerobic power (W)	363 [329-396]	341 [311-371]*	348 [318-378]*		357 [324-390]			359 [327-391]
Mean difference [95% CI]		-21 [7-36]	-15 [2-28]		-6 [-10-23]			-3 [-7-15]
4-minute self-paced trial								
Mean power (W)	300 [234-366]	280 [224-346]*	278 [210-346]*		288 [222-254]			286 [222-350]
Mean difference [95% CI]		19 [2-37]	22 [1-43]		11 [-5-29]			14 [-6-33]
Oxygen uptake ($mL.kg.min^{-1}$)	43.99 [34.59-53.39]	41.73 [33.37-50.09]*	41.98 [33.83-50.13]		42.29 [33.84-50.74]			43.15 [33.88-52.42]
Mean difference [95% CI]		-2.26 [0.51-4.00]	-2.00 [-0.02-4.04]		-1.70 [-1.76-5.15]			-0.84 [-1.86-3.54]

*Significantly less than baseline ($p \leq 0.05$)

5.4.4 Metabolomics Data

Following data cleaning, 2,645 reproducible metabolite features (1,313 ESI POS and 1,332 in ESI NEG) were subject to statistical analysis. Principle components analysis (PCA) of the total data set showed **(Fig. 5-1)** that the QC samples clustered tightly together indicating no batch-to-batch variation and, the QC variance was much smaller than the sample variance. There were no outliers detected. Of the 2,645 features 152 were putatively identified as unique metabolites. RM-ANOVA was performed to select those metabolites that significantly changed post-intervention (i.e. after removal of ~470 mL of blood). In total 40 putatively identified metabolites were statistically altered (corrected p-value ≤ 0.05); see **Table 5-2**. It is important to note that the presented metabolite annotations are putative and should only be discussed as preliminary annotations until chemical validation using chemical standards and two orthogonal properties (i.e. retention time) as defined by the Metabolomics Standard Initiative [282]. In cases where multiple annotations could be assigned, metabolite ID was given based on chemical properties and being the most probable annotation.

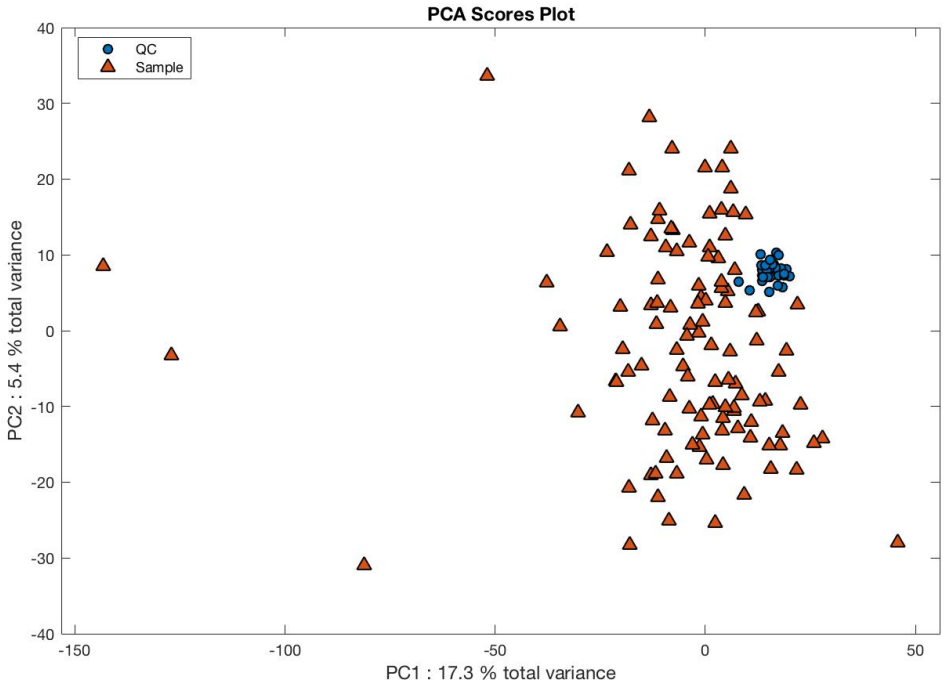

Figure 5-1 Principal components analysis (PCA) scores plot showing all experimental samples of the 2999 reproducible peaks. The quality control (QC) samples (blue circles) formed from pooling small quantities from each sample and tightly cluster in the plot. Red triangles represent samples of each individual obtained at baseline, 24 h and 7 d, 14 d, 21 d, 28 d, 35 d and 42 d after blood removal.

Table 5-2 Putatively annotated metabolic features originating from plasma samples. The table consists of i) the metabolite name, ii) molecular formula, iii) the ANOVA F score, iv) p-value, v) corrected p-value, (vi) cluster group.

Label	Formula	F_time	p_time	q-value	Cluster
Adenosine	C10H13N5O4	2.645	0.016	**0.032**	A
Adenosine monophosphate	C10H14N5O7P	3.772	0.001	**0.004**	A
Guanosine	C10H13N5O5	4.281	0.000	**0.002**	A
Hypoxanthine*	C5 H4 N4 O	2.710	0.013	**0.029**	A
Inosine	C10H12N4O5	4.068	0.001	**0.002**	A
Tetrahydrofolic acid	C19H23N7O6	1.858	0.086	0.108	A
Thymidine 5'-triphosphate	C10H17N2O14P3	3.334	0.003	**0.008**	A
2-Oleoylglycerol	C21 H40 O4	33.570	0.000	**0.000**	B
3-Hydroxyanthranillic acid	C7H7NO3	0.274	0.963	0.475	B
4-Aminohippurate	C9H10N2O3	2.162	0.045	0.070	B
5-Methoxytryptamine	C11H14N2O	0.479	0.847	0.436	B
Arginine*	C6 H14 N4 O2	0.193	0.986	0.477	B
Benzoic acid	C7H6O2	5.900	0.000	**0.000**	B
Caproylglycine	C12 H23 N O3	8.566	0.000	**0.000**	B
Creatine	C4H9N3O2	0.979	0.452	0.299	B
dehydroepiandrosterone sulfate	C19H28O5S	0.651	0.712	0.392	B
Glutamine*	C5 H10 N2 O3	0.839	0.558	0.341	B
Methylmalonic acid	C4H6O4	1.455	0.193	0.186	B
N-Methyl-2-pyrrolidone*	C5 H9 N O	1.522	0.170	0.175	B
N-Undecanoylglycine	C13 H25 N O3	23.695	0.000	**0.000**	B
Nonanoic acid	C9H18O2	5.824	0.000	**0.000**	B
Pantothenic acid	C9H17NO5	0.980	0.451	0.299	B
Pentandioic acid	C5H8O4	1.156	0.336	0.263	B
Resveratrol	C14H12O3	2.230	0.039	0.065	B
Urea	CH4N2O	2.626	0.016	**0.032**	B
Uridine 5'-diphosphoglucuronic acid	C15H22N2O18P2	1.049	0.403	0.285	B
1,3,7-Trimethyluric acid*	C8 H10 N4 O3	0.371	0.917	0.462	C
1,7-Dimethyluric acid*	C7 H8 N4 O3	0.135	0.995	0.477	C
3-Methylxanthine*	C6 H6 N4 O2	0.956	0.468	0.304	C
Caffeine*	C8 H10 N4 O2	0.344	0.931	0.466	C
Gluconic acid	C6H12O7	0.261	0.967	0.475	C
n-acetylserotonin	C12H14N2O2	1.959	0.069	0.095	C
Theobromine*	C7 H8 N4 O2	0.944	0.477	0.307	C
16-hydroxypalmitic acid	C16 H32 O3	5.053	0.000	**0.000**	D
2-Aminoadipate	C6H11NO4	2.196	0.042	0.068	D
2-hydroxybutyric acid	C4H8O3	3.135	0.005	**0.012**	D
25-Hydroxyvitamin D3	C27H44O2	1.434	0.201	0.189	D
Bilirubin*	C33 H36 N4 O6	0.790	0.598	0.356	D
Cholic acid*	C24 H40 O5	1.258	0.280	0.232	D
Citrulline	C6H13N3O3	1.252	0.283	0.232	D
Deoxycholic acid	C24H40O4	1.004	0.434	0.295	D
Dodecanedioic acid*	C12 H22 O4	1.419	0.207	0.189	D
Enterolactone	C18H18O4	1.527	0.168	0.175	D
Estradiol	C18H24O2	2.004	0.063	0.091	D
Folic acid	C19H19N7O6	3.851	0.001	**0.004**	D
Glycocholic acid*	C26 H43 N O6	1.171	0.328	0.260	D
Glycoursodeoxycholic acid*	C26 H43 N O5	0.772	0.612	0.362	D
Homovanillic acid	C9H10O4	0.717	0.658	0.377	D

N-Arachidonoyl Dopamine	C28H41NO3	0.124	0.996	0.477	D
Palmitic acid	C16H32O2	1.988	0.065	0.092	D
Pipecolic acid	C6H11NO2	1.064	0.393	0.281	D
Pyroglutamic acid	C5H7NO3	0.681	0.687	0.384	D
Suberic acid	C8 H14 O4	0.669	0.698	0.387	D
Taurocholic Acid	C26H45NO7S	1.014	0.427	0.295	D
Tetradecanedioic acid	C14 H26 O4	1.937	0.073	0.097	D
Uric Acid	C5H4N4O3	0.644	0.719	0.393	D
Ursodeoxycholic Acid	C24H40O7S	1.496	0.179	0.181	D
11-deoxycortisol	C21H30O4	0.931	0.487	0.310	E
5-Hydroxyindole-3-acetic acid*	C10 H9 N O3	1.004	0.434	0.295	E
5-Hydroxyindoleacetic acid	C10H9NO3	0.563	0.784	0.416	E
AcetylLysine	C8H16N2O3	2.077	0.054	0.081	E
Arabinose	C5H10O5	0.686	0.684	0.384	E
Carnitine*	C7 H15 N O3	1.677	0.124	0.145	E
Ceramide (C24:0)	C42H83NO3	1.893	0.080	0.104	E
Choline	C5 H13 N O	0.998	0.438	0.295	E
Cortisol*	C21 H30 O5	0.706	0.667	0.379	E
Creatinine	C4H7N3O	1.490	0.181	0.181	E
Cytidine 5'-diphosphocholine	C14H26N4O11P2	1.023	0.420	0.294	E
Erythrose 4P	C4H0O7P	0.900	0.510	0.317	E
Fructose 1,6-diphosphate	C6H14O12P2	0.973	0.455	0.299	E
Glucosamine 6-phosphate	C6H14NO8P	0.634	0.727	0.394	E
Hippuric acid*	C9 H9 N O3	2.173	0.044	0.070	E
Histidine	C6H9N3O2	0.376	0.914	0.462	E
Kynurenic acid	C10H7NO3	2.484	0.022	**0.041**	E
Kynurenine	C10H12N2O3	0.811	0.580	0.350	E
Lysine*	C6 H14 N2 O2	0.927	0.490	0.310	E
Methionine*	C5 H11 N O2 S	0.556	0.790	0.416	E
Methyl indole-3-acetate*	C11 H11 N O2	1.357	0.233	0.202	E
myo-Inositol 1,4,5-trisphosphate	C6H15O15P3	2.011	0.062	0.091	E
Myristamide	C14 H29 N O	3.666	0.002	**0.005**	E
N-Acetyl-L-carnosine*	C11 H16 N4 O4	1.353	0.235	0.202	E
Oleic acid	C18 H34 O2	4.301	0.000	**0.002**	E
p-Coumaric acid	C9H8O3	1.234	0.293	0.237	E
Phenylalanine*	C9 H11 N O2	0.754	0.627	0.365	E
Pipecolinic acid*	C6 H11 N O2	1.121	0.357	0.270	E
Proline*	C5 H9 N O2	0.523	0.815	0.426	E
Saccharopine	C11H20N2O6	1.766	0.104	0.127	E
Sphingosine-1-phosphate (18:1)	C18H38NO5P	3.643	0.002	**0.005**	E
Symmetric dimethylarginine*	C8 H18 N4 O2	0.481	0.846	0.436	E
Thymine	C5H6N2O2	3.142	0.005	**0.012**	E
trans-Cinnamic acid	C9H8O2	1.062	0.394	0.281	E
Tryptophan*	C11 H12 N2 O2	1.346	0.238	0.202	E
Tyrosine*	C9 H11 N O3	0.900	0.510	0.317	E
Valine*	C5 H11 N O2	0.867	0.536	0.330	E
Xanthine	C5H4N4O2	2.568	0.018	**0.035**	E
1-[(8Z,11Z,14Z)-icosatrienoyl]-sn-glycero-3-phosphocholine	C28 H52 N O7 P	4.497	0.000	**0.001**	F

1-heptadecanoyl-sn-glycero-3-phosphocholine	C25 H52 N O7 P	3.479	0.002	**0.006**	F
3-Hydroxykynurenine	C10H12N2O4	1.718	0.115	0.138	F
4-Aminophenyl b-D-galactopyranoside	C12H17NO6	0.053	1.000	0.477	F
Adenosine 3',5' cyclic monophosphate	C10H12N5O6P	2.287	0.034	0.059	F
Aldosterone	C21H28O5	3.471	0.002	**0.006**	F
Androst-4-en-3-one	C19 H28 O	3.785	0.001	**0.004**	F
Dihydrotestosterone	C19H30O2	0.571	0.778	0.415	F
Glycerophospho-N-palmitoyl ethanolamine*	C21 H44 N O7 P	1.670	0.126	0.145	F
Indole-3-acetic acid*	C10 H9 N O2	1.372	0.227	0.199	F
Indole-3-lactic acid*	C11 H11 N O3	1.953	0.070	0.095	F
Lyso-PAF C16	C24H52NO6P	3.022	0.007	**0.015**	F
Lyso-PAF C18	C26H56NO6P	2.282	0.035	0.059	F
myo-Inositol	C6H12O6	1.110	0.363	0.270	F
N-Phenylacetylglutamine*	C13 H16 N2 O4	0.286	0.958	0.475	F
Nicotinuric acid	C8H8N2O3	1.128	0.353	0.270	F
Propionylcarnitine*	C10 H19 N O4	0.616	0.741	0.399	F
Shikimic acid	C7H10O5	1.473	0.187	0.182	F
Thiamine	C12H17N4OS	0.394	0.904	0.462	F
trans-Urocanic Acid	C6H5N2O2	1.685	0.122	0.145	F
Tricaprylin	C27H50O6	2.149	0.046	0.071	F
Uridine	C9H12N2O6	2.702	0.014	**0.029**	F
Uridine 5'diphospho-N-acetylglucosamine	C17H27N3O17P2	1.414	0.209	0.189	F
α-Aminocaprylic acid*	C8 H17 N O2	0.757	0.625	0.365	F
2-Hydroxyglutaric acid	C5 H8 O5	11.450	0.000	**0.000**	G
2-Ketobutyric acid	C4H6O3	4.056	0.001	**0.002**	G
3-Hydroxyisovalerylcarnitine	C12 H23 N O5	76.775	0.000	**0.000**	G
5-Hydroxy-L-tryptophan	C11H12N2O3	4.648	0.000	**0.001**	G
cis-11-Eicosenoic acid	C20H38O2	4.413	0.000	**0.001**	G
Heptanoylcarnitine	C14 H27 N O4	11.037	0.000	**0.000**	G
N-formylkynurenine	C11 H12 N2 O4	4.080	0.001	**0.002**	G
Pentonic acid	C5 H10 O6	5.439	0.000	**0.000**	G
Tiglylcarnitine	C12 H21 N O4	10.819	0.000	**0.000**	G
11,14-eicosadienoic acid	C20H36O2	1.420	0.207	0.189	H
12-Hydroxyeicosatetraenoic acid	C20H32O3	1.097	0.372	0.274	H
13-Hydroxyoctadecadienoic acid	C18H32O3	1.123	0.356	0.270	H
3-Hydroxydecanoic acid	C10 H20 O3	1.413	0.210	0.189	H
5-Methoxytryptophol	C11H13NO2	1.564	0.156	0.173	H
5-pregnen-3B-ol-20-one	C21H32O2	1.532	0.166	0.175	H
Acetyl-L-carnitine*	C9 H17 N O4	1.823	0.092	0.115	H
Arachidonic acid	C20H32O2	1.568	0.155	0.173	H
Citric acid*	C6 H8 O7	1.378	0.224	0.199	H
Decanoylcarnitine (C10:0)	C17H33NO4	1.543	0.163	0.175	H
Dodecanoate	C12H24O2	6.076	0.000	**0.000**	H
Elaidic Acid	C18H34O2	1.077	0.385	0.280	H
Linoleic Acid	C18H32O2	1.264	0.277	0.232	H
Linolenic acid	C18H30O2	1.546	0.162	0.175	H
Myristic acid	C14H28O2	3.815	0.001	**0.004**	H
Myristoleic acid	C14H26O2	2.442	0.024	**0.044**	H

Oleoylcarnitine (C18:1)	C25H47NO4	2.627	0.016	**0.032**	H
Palmitoleic acid	C16H30O2	0.810	0.582	0.350	H
Palmitoylcarnitine (C16:0)	C23H45NO4	1.117	0.359	0.270	H
Progesterone	C21H30O2	1.872	0.083	0.107	H
Prolylleucine*	C11 H20 N2 O3	0.729	0.648	0.374	H
Retinol	C20H30O	1.473	0.187	0.182	H
Testosterone	C19H28O2	1.170	0.328	0.260	H

Note: Significance was based on q-value (FDR-adjusted p-values) and significance set at $q \leq 0.05$. All significant metabolites are bolded. * Indicates MS/MS spectra match to the mzCloud library.

The HCA dendrogram (**Fig. 5-2)** grouped metabolites with similar post-intervention metabolite trajectories into eight clear metabolite clusters (labelled A to H). Each of these eight clusters demonstrates a unique trajectory from pre-intervention through to 42 d. Four of the clusters showed significant group-trajectory characteristics. The metabolites in Cluster A (n=7) describe an immediate increase post blood removal (24 h) and a gradual return to pre-intervention levels by 42 d. The metabolites were identified as originating from the purine metabolite pathway. Cluster B (n=19) followed a steady decrease in metabolite concentration with no recovery. Significant metabolites in this group were: Urea, Benzoic Acid, Nonanoic Acid, and minor metabolites of fatty acids. Cluster H (n = 24) demonstrated a delayed recovery trajectory, where no significant change in metabolite concentration occurred until 20 d, followed by a sharp increase until 35 d and then a return to normal levels at 42 d. This cluster consisted of mainly classes of fatty acid metabolites. Finally, Cluster-G (n=9) shows a similar delayed response, but with a steep increase observed from 7 d to 14 d, continuing to 35 d before a slight decrease at 42 d. These metabolites were identified as carnitines and tryptophan metabolism. Clusters C, D, E and H displayed erratic behaviour in which no clear trajectory characteristics can be discerned; however, there were individual metabolites in clusters D, E and F, that displayed significantly changing trajectories. Cluster C, included caffeine metabolites and many derivatives and was observed as non-significant throughout the 42 d tracking.

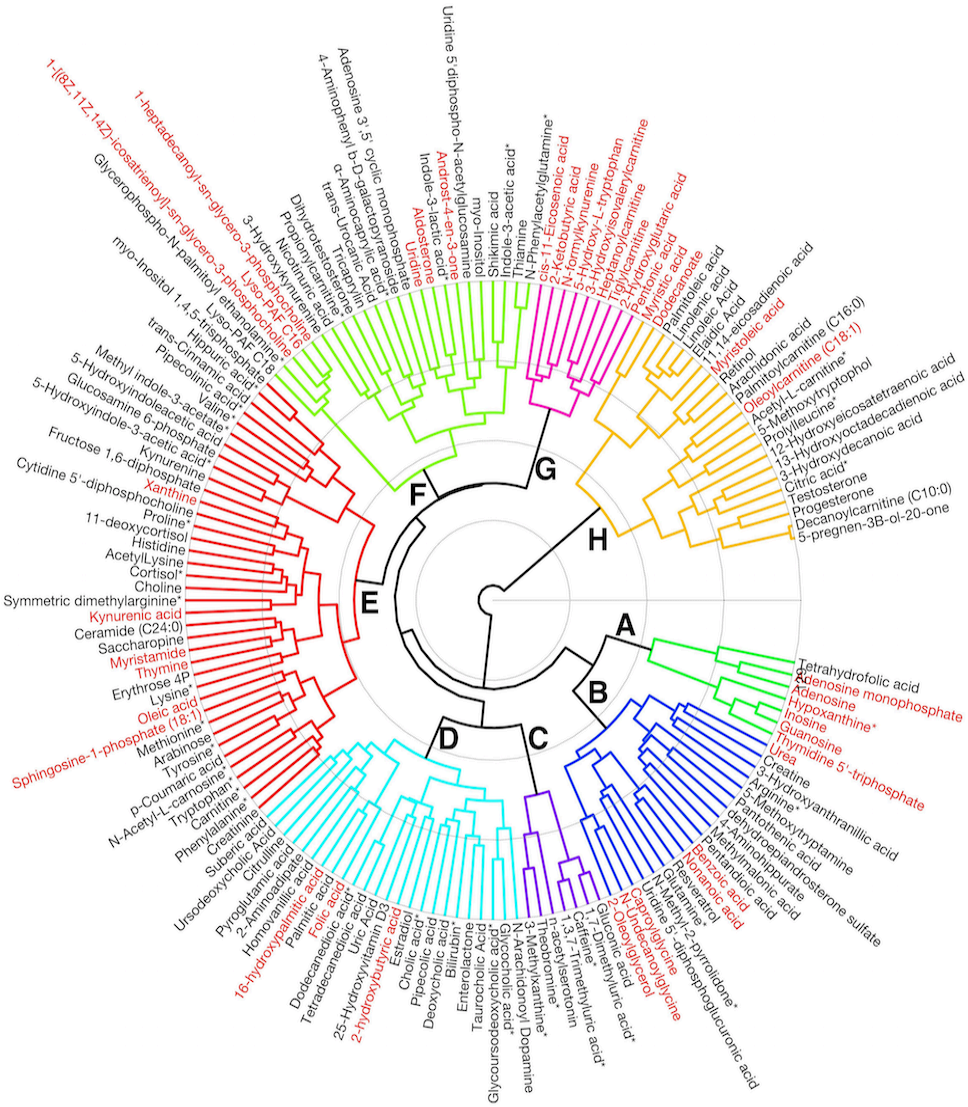

Figure 5-2 Hierarchical cluster plot showing dendrograms (A-H) and showing the relationship between putatively identified metabolites originating from plasma samples. Relation between metabolites is indicated by the length/distance. Metabolites labelled red ($p < 0.05$) and black ($p > 0.05$).

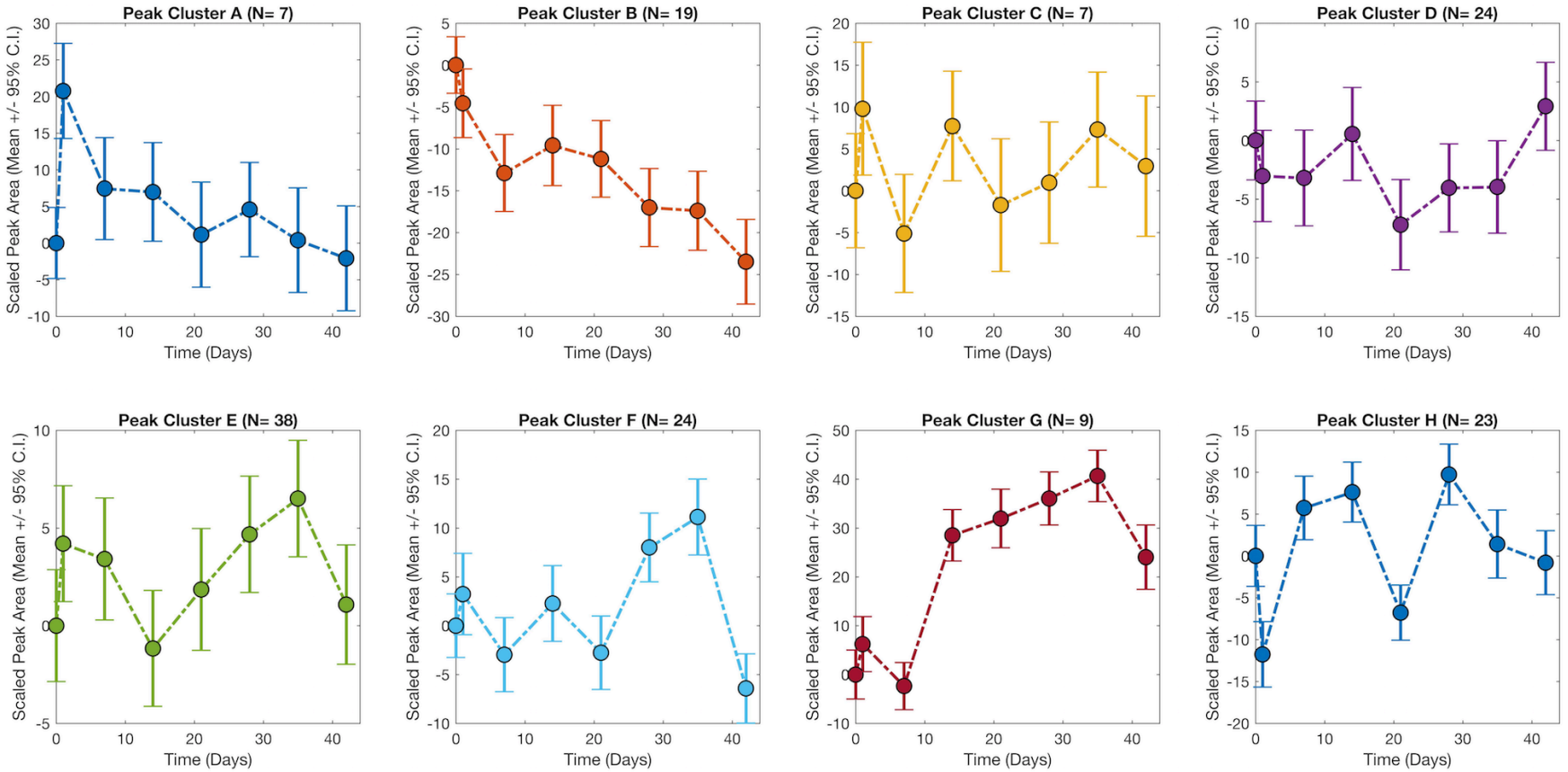

Figure 5-3 Trajectory plots taken from the Hierarchical cluster analysis. N = number of metabolites contributing to the cluster.

5.5 Discussion

The current study examined the impact of blood donation (~470 mL) on acute (i.e. 24 h) and chronic (up to 42 d) changes to metabolic, physiological, haematological and performance measures in recreationally trained males. The main outcomes were; 1) blood removal decreased [Hb] and Hct % until day 14, 2) $\dot{V}O_{2max}$ and performance decreased acutely post-blood removal and demonstrated different patterns of recovery, 3) using an untargeted metabolomics approach, eight key clusters of metabolites were identified with four clusters demonstrating protracted, or non-recovery by 42 d and 4) purine metabolites increased immediately post-blood removal with a gradual recovery to baseline levels by 42 d.

Acutely (24 h) following blood removal, we observed a decrease in Hct % (-9 %) and a reduction in $\dot{V}O_{2max}$ (-6 %), maximal aerobic power (-6 %), and 4MMP power output (-7 %). These findings are not unexpected [198, 199], and have been outlined in a recently published meta-analysis by Van Remoortel, De Buck [117]. Furthermore, and consistent with our previously published sub-group analyses [311], $\dot{V}O_{2max}$ had returned to baseline values by the 7th d while maximal aerobic power and the 4MMP mean power output remained below baseline values. These findings support the hypothesis that immediate changes in $\dot{V}O_{2max}$ and performance after blood removal are likely modulated by central limitations (i.e. oxygen availability) [91, 146], whereas continued decrements in performance are less associated with oxygen availability and therefore mediated by additional factors. Of note, the addition of six moderately trained individuals in this cohort resulted in the recovery of 4MMP performance by 21 d, a finding not previously observed [311]. As fitness levels increase, the capacity to consume oxygen during exercise become more delivery dependent [81, 312]. As such, the addition of lesser trained individuals to this cohort likely diminished the influence of changes in oxygen delivery during later time points.

As hypothesised above, it is likely that additional factors beyond oxygen availability influence performance immediately following blood removal and during the normal red blood cell maturation phase (~42 d). As such, a major focus of this study was to examine changes in the metabolic profile after blood removal. This was achieved through the use of untargeted metabolomic analysis to detect intact small molecules over a wide concentration. From this, we identified 152 putatively annotated metabolites (**Table 5-2**), of which 40 demonstrated significant change following the removal of whole blood (**Fig. 5-2**). It should be noted that final confirmations of metabolite identifications with standards are still needed. Furthermore, it is possible that not all 40 metabolites demonstrating change in this study are of interest. For instance, 'Cluster G' (**Fig. 5-2**) demonstrated several significant metabolites; however, it was not possible to identify a central pathway from our data as each identified metabolite were consistent with

multiple physiological pathways. Future studies employing larger metabolite libraries and a control group will help narrow down the metabolites in this list.

In the absence of clear metabolite identification through comparison with standards, we performed an unsupervised HCA to classify metabolites into similar response groups from which eight sub-cluster trajectories were identified (**Fig. 5-2**). Of interest, cluster-A metabolites demonstrated an immediate and significant increase with a prolonged return towards baseline over the 42 d (**Fig. 5-3**). Within this cluster we identified five metabolites as nucleotides and nucleosides; inosine, hypoxanthine, adenosine, adenosine monophosphate and guanosine, of which all are associated with the purine metabolic pathway. Little is known regarding the influence of blood removal on nucleotides; however, during hypoxic exposure or severe energy depletion, these metabolites have been shown to be important in energy utilisation [176, 270]. From this study, we cannot determine what influence changes in purine metabolism had on our fitness and performance data; however, in animal studies, dietary supplementation with nucleotides including inosine and guanosine have been shown to increase levels of 2,3-diphosphoglycerate [176, 177]. An increase in 2,3-diphophoglycerate can result in a rightward shift of the oxygen dissociation curve [313]; thus, reducing the affinity of haemoglobin to oxygen. It is possible that in the present study, purine metabolism under decreased oxygen availability (i.e. reduced Hct% and [Hb]) could have acted in a compensatory manner to increase levels of 2,3-diphosphglycerate thereby enhancing oxygen delivery to the periphery. This hypothesis supports our observation that both $\dot{V}O_{2max}$ and $\dot{V}O_2$ during the 4MMP test returning to baseline values at 7 d, during a period when red blood cell regeneration would have been incomplete [197].

This study provides import insight into the influence of blood donation on the metabolic changes immediately following and up to 42 d post blood removal. However, there are some noted limitations to the present study. Although activity monitors were worn for the duration of the study, it is important to acknowledge that a limitation of such measure is they do not provide information on non-ambulatory activity (i.e. resistance training or swimming), intensity or type of physical activity. Another limitation to this study was the influence of diet on metabolite findings. As such participants dietary choice during the 42 d could influence the metabolome [314]. However, it should be noted participants were asked to replicate their diet as close as possible to their previous trials and undertook 8 h fast before the experimental trials which, is a level of control consistent within the metabolomic literature [260, 315].

5.6 Conclusions

In conclusion, the removal of 470 mL of whole blood acutely (24 h) impacts oxygen carrying capacity and exercise performance. However, differences in the recovery of $\dot{V}O_{2max}$ and performance suggest that these two measures are not intrinsically linked. The recovery of $\dot{V}O_{2max}$

did not coincide with the recovery of [Hb] indicating possible compensatory mechanisms, such as changes in purine metabolism, occur to enhance oxygen availability to working muscles. Without the analytical standards necessary to confirm the identity of all metabolites, these finding should be viewed as preliminary until more definitive identification can occur.

Chapter 6 Thesis summary

Historically the measurement of an individual's maximal ability to consume oxygen ($\dot{V}O_{2max}$) has been used as a predictor of endurance performance [51, 52]. As such, training strategies such as hypoxic exposure have been used with the intent of increasing oxygen carrying capacity and therefore endurance performance [45]. While an increase in RBCs is regarded as the critical response to altitude training through the up-regulation of erythropoietin [30, 130, 164], many other unexplored changes have been demonstrated to occur during this time [46]. For instance, non-haematological changes occur with improvements in exercise economy, skeletal muscle buffering and cellular adaptations such as HIF -1α, as well as changes to aerobic and anaerobic enzyme concentration [31, 46]. Therefore, it is recognised that changes in Hb_{mass} are not solely responsible for changes in performance [46, 47]. Given the possible benefits, the non-haematological mechanisms warrant further examination.

Metabolomics has a history of use in human biological samples [34, 307, 308] and more recently in the area of exercise sciences [35, 246, 260, 263, 309]. This technique provides the ability to comprehensively characterise system level changes in metabolism at a given time point [207], thus providing a unique metabolite profile. The use of techniques consistent with metabolomics (GC-MS, LC-MS and NMR) can help to better understand the physiological response to hypoxia by providing the ability to monitor systemic changes through the construction of metabolite profiles. Limited metabolite profiling research has been conducted to explore altitude exposure in athletes, and to the authors knowledge, metabolite profiling is yet to be used to explore the use of blood donation as a hypoxic model. Therefore, the purpose of the thesis is to explore low oxygen carrying capacity and its influence on fitness and performance as well as describe the acute and chronic physiological and biochemical adaptions to low oxygen carrying capacity. This chapter provides a summary of the important results from the experimental chapters of this thesis and integration of the findings. Finally, this chapter concludes with an assessment of the thesis findings resulting in the identification and rationalisation of future research.

Chapter Three was conducted as a component of a larger observational study investigating the influence of pre and post-exercise hepcidin levels in well-trained runners during a 14 d altitude camp [16]. Specifically, Chapter Three utilised metabolomics to examine the acute metabolite profiles in response to 14-d moderate altitude exposure (~3000 m). This analysis demonstrated separation in metabolite profiles (173 metabolites) at the three sampled time points (baseline, 3 d and 14 d), indicating a temporal association with outcome measures. From the original 176 metabolites, 36 were identified from metabolite classes including amino acids, glycolysis and purine metabolism. These findings are consistent with previous literature [31, 49] and indicate a shift in substrate utilisation (i.e. greater carbohydrate use) during acute hypoxic

exposure. Through further analyses using CVA, the correlated structure of the 36 metabolites and their associated trajectories were identified, with two different trajectories observed during the acute moderate altitude exposure. The first trajectory revealed metabolites decreasing in the early phase of altitude exposure (3 d) that returned to baseline levels by 14 d. The second set of metabolites demonstrated either an increase or decrease in concentration upon altitude exposure and did not return to baseline levels by 14 d. The metabolites identified in the two trajectory sets warrant further investigation, as it is possible that they could help to further explain the acute metabolic changes associated with altitude exposure [49, 269]. In addition to differences in the metabolic profiles, analyses of the PCA plots demonstrated greater between-person compared with within-person variance. These findings provide further support to previous literature suggesting a high level of individual variability during the adaptive response to altitude exposure [47, 269].

Chapter Three indicates that acute metabolic adaptations occur in response to a decrease in oxygen carrying capacity during short-term hypoxic exposure. Nevertheless, several potential limitations have been identified that may have influenced data collection and the interpretation of the results. This study was a collaboration with the Australian Institute of Sport during a predetermined altitude training camp. The use of athletes (sample of convenience), while providing an elite calibre population, did result in a relatively small sample size and therefore affected the study's statistical power. While every effort was taken to ensure a similar level of control during the altitude camp to reduce variability on the blood metabolome, inevitable some variables were outside of the researcher's control (i.e. recording of diet and physical activity). It is possible that some metabolic changes identified during this study were the consequence of training and diet, and not directly due to altitude exposure [316-318]. Finally, due to scheduling restrictions, the use of the Australian Institute of Sport altitude facilities was limited to 14 d. The findings in Chapter Three therefore only represent acute metabolic changes associated with altitude exposure, as a greater measurement period (up to ~42 d) would be needed to assess full acclimatisation (i.e. metabolic and haematological change; [15]).

In Chapters Four and Five, Australian Red Cross blood donation (~470 mL) was used to induce a reduced oxygen carrying capacity in 13 male participants. This allowed the ability to examine the adaptive processes occurring over the course of normal red blood cell maturity (~42 d). During this study, measures of [Hb] and Hct % were obtained before blood removal (baseline) and at seven time points (24 h, 7, 14, 21, 28, 35 and 42 d) following blood removal to indicate oxygen carrying capacity (Chapters Four and Five). Additionally, exercise tests were performed to assess fitness ($\dot{V}O_{2max}$) and performance through a 4-min self-paced cycling test (4MMP) Chapters Four and Five, as well as the collection of a 4 mL blood sample for subsequent metabolomic analysis (Chapter Five). Importantly, as purine metabolism was identified as an area of interest in Chapter Three, this was a subsequent area of focus for Chapter Five.

Performance during middle-distance endurance events is reliant on aerobic metabolism [43], while pacing during these events has been associated with the conservation of anaerobic energy stores [68, 319]. Furthermore, consistent with previous research [288, 320] and data from Chapter Three, acute hypoxic exposure results in a greater reliance on anaerobic metabolism. Thus, in Chapter Four the aim was to determine the influence of blood donation on pacing and energy resources during self-paced middle distance cycling in trained cyclists. Seven of the 13 participants regarded as 'trained' were used for data analysis. Consistent with previous research [117, 198, 201], [Hb], Hct %, and $\dot{V}O_{2max}$ were reduced up to 7 d following blood donation. However, a novel finding was that 4MMP average power output was reduced up to 21 d, despite the return of [Hb], Hct % and $\dot{V}O_{2max}$. In addition to average power output, pacing during the 4MMP was influenced by the removal of blood. Initial power outputs were reduced over the first 2 min of the 4MMP trials when compared to baseline. Contributing to the differences in individual pacing profiles were corresponding reductions in aerobic power; yet, no significant changes in anaerobic power contributions were observed.

The absence of a change in anaerobic power contribution to the 4MMP after blood removal contradicts findings of a systemic shift to greater reliance on anaerobic metabolism (Chapter Three). For instance, a greater contribution of anaerobic power output would have been expected throughout the 4MMP, post blood donation, especially within the first 14 d. However, the high intensity nature of the 4MMP would require near maximal aerobic and anaerobic energy contribution [69], thus it is possible that although aerobic contribution was reduced, anaerobic contribution was already maximal. Alternatively, athletes may regulate anaerobic energy contribution during middle distance time trials to optimise performance [43]. It is possible that participants in this study could have subconsciously regulated use of anaerobic energy stores, attenuating any increase in anaerobic contribution to the 4MMPs.

The focus of Chapter Five was to investigate metabolic changes immediately following and up to 42 d post blood removal using an untargeted metabolomic approach. In this chapter, an additional six participants (i.e. recreationally untrained) were added to the seven 'trained' participants in Chapter Four to increase robustness of the statistical analysis. To the authors knowledge, no evidence currently exists to indicate differential adaptive process to hypoxia between trained and untrained individuals. Following blood donation, a decrease in [Hb], Hct % and (4MMP) $\dot{V}O_2$ was observed, however changes in $\dot{V}O_2$ only persisted for 24 h when analysed as a combined group (n = 13). This finding was unexpected, as the same measures reported in the subset of trained males lasted 21 d (Chapter Four). The accelerated return in $\dot{V}O_2$ was likely associated with participant fitness level (i.e. $\dot{V}O_{2max}$: 53.0 ± 10.9 $mL \cdot kg^{-1} \cdot min^{-1}$; (Chapter Five) vs. $\dot{V}O_{2max}$: 60.7 ± 5.5 $mL \cdot kg^{-1} \cdot min^{-1}$ (Chapter Four)), as in this case the addition of lesser trained individuals seemingly diminished the influence of oxygen delivery in the later time points [10,

273, 321, 322]. Importantly, changes in $\dot{V}O_2$ and performance were not consistent with one another, providing additional evidence to support that fitness and performance are not intrinsically linked [323].

Metabolomic analysis using mass spectrometry were conducted on 4 mL whole blood samples obtained before blood removal (baseline) and seven time points following blood removal (24 h, 7, 14, 21, 28, 35 and 42 d). From this data, multi-factorial changes were identified following blood donation, with n = 40 metabolites deemed significant. Hierarchical cluster analysis revealed eight metabolite cluster trajectories. Importantly, and consistent with findings in Chapter Three, one of the eight metabolite cluster trajectories were associated with purine metabolism. Furthermore, the trajectory of this cluster remained elevated, although trending back to baseline, through the 42 d monitoring period. These results indicate that purine metabolism influences both the acute (Chapter Three) and chronic adaptive phases (Chapter Five) of hypoxia. Therefore, it is possible that purine metabolites could be used as a marker of reduced oxygen carrying capacity and the adaptive responses of the body to chronic hypoxia such as altitude training.

The findings from this thesis provide important insights into performance and metabolite changes to hypoxia, however, it is important to acknowledge some study limitations could potentially influenced our findings. Chapter 3 was an observational study and unfortunately provided several limitations. Firstly, it was not possible to implement a control group into the altitude camp study. As such, some of our metabolite findings may not be unique to altitude exposure and possible influenced by other external factors. However, the measured responses of EPO and Hb_{mass} [16] were consistent with previous published LHTL studies and demonstrate an altitude adaption occurred [13, 15]. Another limitation of this study was the inability to analyse athletes' training volume or dietary intake as covariates in our statistical model. As such, differences in athletes' training volume during the LHTL could have confounded physiological adaptions to altitude. However, during the study athletes were instructed to maintain their normal diet and training program, therefore differences may account for some between-athlete variation in metabolic profiles.

In Chapters 4 and 5 participants reported to each experimental session following similar dietary consumption to the previous experimental session and fasted for 8 h at the same of day which can acutely control for dietary influence on performance and blood metabolites [324, 325]. However, the influence of chronic dietary intake (i.e. 42 d) was not analysed and subsequently was not included as a covariate in the statistical models. Previously, Walsh et al. (2006) suggested that consumption of a standardised diet before blood sampling may reduce inter-participant variations, as differences in diet may cause metabolic changes that may be difficult to differentiate from normal physiological variations [314]. Therefore, it is possible participant dietary choices influenced haematological regeneration following blood removal, which in turn could have

influenced metabolite response. Another key limitation in Chapter 4 and 5 lies in the ability to understand training load on performance and the blood metabolome. While activity monitors were provided to participants to measure daily activity levels (EPOCHs), a significant limitation of this measure is the inability account for non-ambulatory physical activities (i.e. resistance training) and intensity of activities which could influence a participants overall training load and thus may have influenced the outcome of the performance trials.

In summary, the metabolomics approach in Chapter Three revealed separation in plasma metabolite profiles during a moderate altitude camp. Importantly, the findings indicated anaerobic contribution was likely compensating for the hypoxic condition. Using blood donation to decrease oxygen carrying capacity in Chapter Four, anaerobic energy contribution did not compensate for the removal of 470 mL blood as measured by power output during a 4MMP trial; however, this finding was likely a consequence of the exercise intensity and intrinsic strategy to maintain anaerobic energy stores. When examined over 42 d, hypoxia did result in metabolic disturbances, most notably an increase in purine metabolism. Importantly, purine metabolites could provide a robust marker for the adaptive process following hypoxic exposure.

6.1 Directions for future research

The results from this thesis provide support for the use of metabolomics to explore the influence of low oxygen carrying capacity in individuals. However, more research is required to further our understanding of the resultant biochemical changes. By understanding the impact of low oxygen carrying capacity on an individual, recommendations on the use of altitude training may be possible. For example, in Chapter Three, PCA revealed a greater degree of separation between participants when compared to all time points. This finding is interesting and indicates hypoxic responses are individualised, providing further insights into the intra- and inter-individual differences. To this end, further investigation of these metabolites during moderate altitude exposure may help to more accurately describe the complex physiological and metabolic adaptions that occur in athletes during moderate altitude exposure.

While this thesis was not designed for anti-doping research, the procedures and findings of these chapters can provide support to the athlete biological passport (ABP). Currently, the integrity of the ABP is formed through the understanding of normal fluctuations of biomarkers to assess an athlete for a doping offence [326]. However, environmental changes such as altitude may produce spurious results through variation in the biomarkers used in the haematological model. As such, low oxygen carrying environments, such as those used in Chapter Three, are regarded as one of the major confounders to the ABP [327]. For these reasons, new robust biomarkers which can indicate low oxygen carrying environments could help explain when athletes have been exposed to hypoxia and may be beneficial in advancing the ABP model.

Although not possible in this thesis, including a placebo to investigate the effects of blood donation on pacing would be of interest. Although pacing was altered following blood donation (Chapter Four), the mechanisms behind the change could not be accurately determined. To further investigate the influence of low oxygen carrying capacity on pacing, the use of a placebo, such as an opaque line during blood removal can further understanding of physiological mechanisms vs. psychological change, which may be attributed to the change in pacing strategy (Chapter Four).

While Chapter Five provided an in-depth analysis of the metabolic changes associated with blood donation, future work is necessary to confirm metabolite identifications. Further studies are needed to establish the role of purines in athletes during hypoxia. This could be achieved through purine supplementation and through the development of a targeted analytical method to quantify the change in metabolite concentration. Both Chapters Three and Five only measured metabolites which were amenable to C18 chemistry. Future studies should include hydrophilic interaction chromatography (HILIC) to provide comprehensive metabolome coverage (i.e. separation of small polar metabolites). It would be interesting to determine metabolic pathways associated with identified purine metabolites. This would require implementing different analytical methods to capture metabolites which surround the purine pathway, such as using HILIC to measure ATP. Using HILIC is complimentary to C18, and it can provide two strengths: i) confirming already identified metabolites, and ii) providing greater coverage of the metabolome through retention of polar metabolites which are more amenable to HILIC chemistry.

Finally, metabolite identification yield was increased in Chapter Five, through the use of the Thermo Scientific Q-Exactive™ Orbitrap and mzCloud repository, it is strongly suggested at minimum a library of metabolites of interest and associated fragmentation patterns is generated to improve accuracy of identification.

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Human Kinetics
ORIGINAL INVESTIGATION

The Influence of Blood Removal on Pacing During a 4-Minute Cycling Time Trial

Nathan G. Lawler, Chris R. Abbiss, Aaron Raman, Timothy J. Fairchild, Garth L. Maker, Robert D. Trengove, and Jeremiah J. Peiffer

Purpose: To examine the influence of manipulating aerobic contribution after whole-blood removal on pacing patterns, performance, and energy contribution during self-paced middle-distance cycling. ***Methods:*** Seven male cyclists (33 ± 8 y) completed an incremental cycling test followed 20 min later by a 4-min self-paced cycling time trial (4MMP) on 6 separate occasions over 42 d. The initial 2 sessions acted as familiarization and baseline testing, after which 470 mL of blood was removed, with the remaining sessions performed 24 h, 7 d, 21 d, and 42 d after blood removal. During all 4MMP trials, power output, oxygen uptake, and aerobic and anaerobic contribution to power were determined. ***Results:*** 4MMP average power output significantly decreased by 7% ± 6%, 6% ± 8%, and 4% ± 6% at 24 h, 7 d, and 21 d after blood removal, respectively. Compared with baseline, aerobic contribution during the 4MMP was significantly reduced by 5% ± 4%, 4% ± 5%, and 4% ± 10% at 24 h, 7 d, and 21 d, respectively. The rate of decline in power output on commencement of the 4MMP was significantly attenuated and was 76% ± 20%, 72% ± 24%, and 75% ± 35% lower than baseline at 24 h, 21 d, and 42 d, respectively. ***Conclusion:*** Removal of 470 mL of blood reduces aerobic energy contribution, alters pacing patterns, and decreases performance during self-paced cycling. These findings indicate the importance of aerobic energy distribution during self-paced middle-distance events.

Keywords: self-paced, performance, anaerobic capacity, aerobic power

The influence of blood manipulation on exercise performance is complex and likely associated with both hematological and nonhematological alterations that influence aerobic and anaerobic metabolism.[1] For instance, enhancing oxygen delivery through increasing hemoglobin mass via chronic altitude exposure,[2–4] red blood cell infusion,[5,6] or erythropoietin administration[7] can increase aerobic capacity and exercise performance, whereas reductions in plasma volume and hemoglobin mass after the removal of blood,[8–12] the partial blockade of oxygen binding with hemoglobin through carbon monoxide administration,[13] and decreasing arterial oxygen content through acute altitude-induced hypoxemia[14] can compromise maximal aerobic capacity ($\dot{V}O_{2max}$) and exercise performance. This influence may be especially apparent in middle-distance events, which are characterized by a high reliance on aerobic metabolism and where a high level of aerobic fitness is common.[15]

Despite the importance of $\dot{V}O_{2max}$ to middle-distance events, it is possible that anaerobic energy stores may have a considerable influence on self-selected pacing. Indeed, it has been hypothesized that individuals will manipulate pace throughout an event to spare their anaerobic energy reserve for the final 10% to 15% of the effort.[15] Manipulation of diet[16,17] (eg, caffeine intake) and motivation[18] have also been shown to augment the distribution of anaerobic energy contribution, resulting in altered pacing and improving performance during middle-distance events. Due to the high aerobic demand during middle-distance events, it is plausible that reducing aerobic delivery through decreasing blood volume may alter overall pacing through an increased reliance on anaerobic metabolism. However, to date, the influence of altering aerobic energy contribution on the distribution of energetic resources and pacing during middle-distance performance is unclear.

The purpose of this study was to determine the influence of voluntary blood donation on pacing and energy resources during self-paced middle-distance cycling in trained cyclists. We hypothesized that blood removal would decrease $\dot{V}O_{2max}$ and exercise performance, as well as altering the overall pacing profile. We further hypothesized that the changes in hematological measures in the 42 days after the blood removal would result in a gradual recovery in $\dot{V}O_{2max}$, as well as performance and the aerobic contribution, during a self-paced cycling task.

Methods

Participants

Seven trained male cyclists (mean ± SD age 33 ± 8 y, height 1.8 ± 0.1 m, body mass 72.1 ± 6.9 kg, $\dot{V}O_{2max}$ 60.7 ± 5.5 mL · kg^{-1} · min^{-1}, maximal aerobic power 401 ± 38 W) volunteered to participate in this study. Participants had previous experience performing cycling time trials and were regarded as trained based on previous classifications.[19] Individuals with a history of hypertension, taking prescribed medications, or deemed ineligible to donate blood per the Australian Red Cross blood donation questionnaire were excluded from the study. Participants were asked to maintain regular training commitments and record them (hours and distance per week) throughout the duration of the study, to refrain from heavy exercise

Lawler, Raman, Fairchild, and Peiffer are with the School of Psychology and Exercise Science; Maker, the School of Veterinary and Life Sciences; and Trengove, the Separation Science and Metabolomics Laboratory, Murdoch University, Murdoch, WA, Australia. Abbiss is with the Centre for Exercise and Sport Science Research, Edith Cowan University, Joondalup, WA, Australia. Lawler (n.lawler@murdoch.edu.au) is corresponding author.

Appendix B Additional publications

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SCANDINAVIAN JOURNAL OF MEDICINE & SCIENCE IN SPORTS

Live high, train low – influence on resting and post-exercise hepcidin levels

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The post-exercise hepcidin response during prolonged (>2 weeks) hypoxic exposure is not well understood. We compared plasma hepcidin levels 3 h after exercise [6 × 1000 m at 90% of maximal aerobic running velocity (vVO_{2max})] performed in normoxia and normobaric hypoxia (3000 m simulate altitude) 1 week before, and during 14 days of normobaric hypoxia [196.2 ± 25.6 h (median: 200.8 h; range: 154.3–234.8 h) at 3000 m simulated altitude] in 10 well-trained distance runners (six males, four females). Venous blood was also analyzed for hepcidin after 2 days of normobaric hypoxia. Hemoglobin mass (Hb_{mass}) was measured via CO rebreathing 1 week before and after 14 days of hypoxia. Hepcidin was suppressed after 2 (Cohen's d = −2.3, 95% confidence interval: [−2.9, −1.6]) and 14 days of normobaric hypoxia (d = −1.6 [−2.6, −0.6]). Hepcidin increased from baseline, 3 h post-exercise in normoxia (d = 0.8 [0.2, 1.3]) and hypoxia (d = 0.6 [0.3, 1.0]), both before and after exposure (normoxia: d = 0.7 [0.3, 1.2]; hypoxia: d = 1.3 [0.4, 2.3]). In conclusion, 2 weeks of normobaric hypoxia suppressed resting hepcidin levels, but did not alter the post-exercise response in either normoxia or hypoxia, compared with the pre-exposure response.

Endurance athletes commonly undertake 2–4 weeks of exposure to moderate altitude (2000–3000 m) in an attempt to enhance their performance in aerobic events (Gore et al., 2013). Prolonged exposure to moderate altitude has been shown to induce several hematological (i.e., an increased hemoglobin mass) and non-hematological (i.e., increased oxidative and glycolytic enzyme production) benefits (Gore et al., 2007), which may in turn increase oxygen delivery or extraction during exercise. The approximately three-fold increase in erythropoiesis that occurs during the first few days of exposure to moderate altitude places a large burden on the body's iron stores (Faura et al., 1969). Athletes with low pre-altitude iron stores (serum ferritin levels <35 μg/L) (Peeling et al., 2007), or who are unable to rapidly mobilize iron from storage sites during the first few days of hypoxic exposure may therefore be unable to sustain accelerated erythropoiesis at altitude. For example, red cell volume was blunted in iron-deplete (serum ferritin: 15 ± 3 μg/L) but not in iron-replete (serum ferritin: 69 ± 10 μg/L) athletes exposed to 2500 m natural altitude for 4 weeks (Stray-Gundersen et al., 1992). Consequently, maintaining a healthy iron balance during prolonged exposure to moderate altitude ensures athletes can provide sufficient iron to support increased heme and non-heme protein synthesis.

Both low iron stores and poor iron availability potentially compromise heme synthesis and the production of oxidative and glycolytic enzymes involved in energy metabolism (Dallman, 1986). Accordingly, several changes in iron-related hormone production and iron protein expression occur during prolonged exposure to hypoxia to improve iron availability (Chepelev & Willmore, 2011). A key response to terrestrial altitude exposure is the suppression of hepatic hepcidin production within 24–48 h (Goetze et al., 2013). Hepcidin suppression is a favorable early response to prolonged hypoxia, as it acts to increase intestinal iron absorption and iron export

At the time of thesis submission, this section is published in the *Scandinavian Journal of Medicine and Science in Sport*

Govus A.D., Peeling P., Abbiss C.R., **Lawler N.G.,** Swinkels D.W., Laarakkers C., Thompson K.G., Peiffer J.J., Gore C.J., Garvican-Lewis L.A. (2016).

Appendix B represents a separate study conducted in the same study population as in *Chapter Three* to investigate high-intensity interval exercise influences iron metabolism (i.e. hepcidin) in endurance athletes undertaking 14 d moderate altitude exposure. The primary analysis was conducted by Dr Govus, but significant contribution in regard to the experimental procedures and data collection and manuscript revision was provided by the author of this thesis.

Appendix C Informed Consent: Study 1

Murdoch UNIVERSITY

Informed Consent

Examining metabolite and performance changes associated with reduced blood volume.

1. I agree voluntarily to take part in this study.

2. I have read the Information Sheet provided and been given a full explanation of the purpose of this study, of the procedures involved and of what is expected of me. The researcher has answered all my questions and has explained the possible problems that may arise as a result of my participation in this study.

   Particularly, I acknowledge that I understand the following procedures will occur during this study:

   1. I will be asked to participate in the following during an 7-week period:
      a. Completion of six fitness (cycling exercise test) and performance (2km cycling time trial) tests.
      b. Provide 1 unit (470 ml) of blood to the Australian Red Cross.
      c. Provide eleven venous blood samples (one x 10 ml and 10 x 2 ml) a total of 30 ml throughout the 7- week study.
      d. Have my red blood cell mass measured which consists of:
         i. Inhaling a small dose of carbon monoxide.
      e. Accurately record dietary intake on a daily basis.
      f. Wear an Actigraph for measurement of daily activity levels for the duration of the study.

   I also acknowledge that my minimum commitment to this project will be 13 hours over 7 weeks.

3. I understand I am free to withdraw from the study at any time without needing to give any reason.

4. I understand I will not be identified in any publication arising out of this study.

5. I understand that my name and identity will be stored separately from the data, and these are accessible only to the investigators. All data provided by me will be analysed anonymously using code numbers.

6. I understand that all personal information provided by me is treated as confidential and will not be released by the researcher to a third party unless required to do so by law.

7. I understand that all data, including blood samples, obtained during this study will be stored for five years during which time the data may be used in retrospective research related to this project. Furthermore, I acknowledge specifically:

   a. A small sample of my blood will be stored for the duration of 5 years during which it may be used to help develop methods to increase the sensitivity of metabolite detection in blood. .

Signature of Participant: _____ Date: ...../...../.....
(Name)

Signature of Investigator: _____ Date: ...../...../.....
(Name)

Appendix D Information Letter: Study 1

Murdoch UNIVERSITY

Appendix B

www.murdoch.edu.au

Information Letter

Project Title: Examining metabolite and performance changes associated with reduced blood volume.

Investigator (s) **Mr. Nathan Lawler**
Dr. Jeremiah Peiffer
Dr. Timothy Fairchild

Contact Person Mr. Nathan Lawler
Address Murdoch University
90 South Street
Murdoch, WA, 6150
Telephone No. (61 08) 9360 1450

You have been invited to participate in this study as you are:

1. Male between 18 and 44 years old.
2. Abstained from smoking in the last 6 months.
3. Have not been diagnosed with high-blood pressure and or are not currently taking prescribed medications.
4. Are able to commit to the following:
    a. Training intervention 150 minutes weekly for 11 weeks (i.e. 3 x weekly at 50 minutes a session)
    b. Ten experimental testing sessions (six at 120 minutes each and four at 10 minutes)

Furthermore, you consider yourself to:

1. To be low risk for participation in exercise.
2. Be able to donate blood according to the Australian Red Cross Blood Service.

Aim of the Study

The purpose of this study is to analyse the metabolite profile (chemical make-up) of human blood, after the removal of 1 unit (i.e. 470 ml) of blood, over a duration (42 days) consistent with the body's natural regeneration of the removed blood. Additionally, we aim to examine the influence of blood loss and regeneration on exercise performance. These aims will be achieved while monitoring two different groups of participants; 1) a group completing a structured exercise program during the 42 day monitoring period and 2) a group maintaining their current activity levels; yet, receiving no additional training during the 42 day monitoring period.

What Does Your Participation Involve?

As a possible participant in this study, you will be contacted by a member of the research team and asked a series of questions regarding your general health and suitability to provide a blood donation to the Australian Red Cross. Both surveys will be used to determine your eligibility in this study.

Familiarisation Session

If deemed eligible, you will be asked to attend a familiarisation session at the Murdoch University Exercise Physiology laboratory. During this visit a 10 ml venous blood sample (similar to giving blood at a pathology centre or doctor's office) will be taken from your arm by a trained phlebotomist.

Baseline Testing

Approximately one week after the familiarisation session, you will be asked to return to the laboratory to undergo a fitness (cycling exercise test; approximately 15 minutes) and performance (cycling 2 km as fast as you can) test, provide a 2 ml venous blood sample and have your total haemoglobin mass (portion of the blood that carries oxygen) measured. To measure your total

haemoglobin mass, you will be asked to breathe in a small, safe quantity of carbon-monoxide. This session will take approximately 120 minutes.

Group allocation

You will be randomly allocated into either:

1) Exercise group, who will undertake a structured exercise program.
2) No exercise group, who will not be prescribed an exercise program.

The exercise group will be asked to commence a training program no greater than one week after completing the baseline testing session. You will be asked to attend the Murdoch University Exercise Physiology laboratory three times a week for a supervised 50-minute stationary cycling session. In the first four weeks, your exercise intensity will be gradually increased each week until you are at the desired training intensity, which will be maintained for the remainder of the study. The no exercise group will be asked to maintain their daily activity; yet, will not be asked to participate in any structured training program at any time throughout the study. Regardless of group allocation, you will be asked to wear an activity monitor for the duration of the study, which will record your daily physical activity levels and you will be asked to keep a daily food diary.

Additional Experimental Sessions

All participants (regardless of group allocation) will be asked to visit Murdoch University for nine measurement sessions after baseline testing. On days 35, 37, 43, 57, and 78 post baseline testing, participants will be asked to complete a series of tests identical to the baseline session. On days 39, 50, 64 and 71 after baseline, you will be asked only to provide a 2 ml venous blood sample. The blood sampling sessions should take no longer than 10 minutes.

Red Cross Blood Donation

36 days after baseline testing, all participants will be asked to attend the Australian Red Cross blood donation centre (i.e. time of convenience) and will be asked to donate 1 unit (470 ml) of blood. This blood will not be used in this study and will be donated to the Australian Red Cross. Following your blood donation, you will be monitored and provided with refreshments for 30 minutes before being able to leave the donation centre.

As a routine component of your Australian Red Cross blood donation, your blood will be screened for health related factors, which would influence your ability to donate blood (i.e. ensuring blood safety). All information collected by the Red Cross will be confidential and only discussed with yourself. If you are notified of any abnormalities, you may wish to withdraw from the study and can do so at any time.

Participant Time Commitment

The researchers in this study recognise your participation will require a large time commitment. Regardless of your group allocation, as a participant, you will be asked to take part in approximately 13 hours (includes the familiarisation session) of data collection during the eleven week study. Individuals allocated to the exercise group will be asked to attend three training sessions a week (i.e. 150 min/wk) which in total will equate to an additional 27.5 hours of participation.

Blood Collection

As a participant of this study you will asked to donate 1.0 unit (470 ml) venous blood to the Australian Red Cross. This is a vital process for this study to be conducted. Additionally, throughout this study you will be asked to provide venous blood samples for a total of ten samples (30 ml in total). These samples will be used to measure the metabolite profile of your blood at each time point. After each blood draw, you will be provided the opportunity, if needed, to rest up to 30 minutes before starting any exercise or leaving the laboratory. These samples are an extremely

important part of this study; thus it will be necessary that you are prepared to provide all ten blood samples.

Voluntary Participation and Withdrawal from the Study

It is important that you understand that your involvement is this study is voluntary. While we would be pleased to have you participate, we respect your right to decline. If you decide to discontinue participation at any time, you may do so without providing an explanation. If you withdraw, all information you have provided will be destroyed. All information will be treated in a confidential manner, and your name will not be used in any publication arising out of the research. All of the research will be kept on a password protected computer not accessible by non-research staff.

Possible Benefits

Participants will receive multiple maximal graded exercise tests. From these tests, participants will be provided with accurate and current aerobic fitness values. Furthermore, participants will be provided with information regarding daily activity levels and dietary habits. The data provided to participants can be used as an indication of individual fitness level and further, aid as a guide towards training intensities for maintaining ideal fitness.

If you are selected to participate in the exercise group, you will be provided with an 11 week monitored training program. It is expected that participation in such a program will result in an increase in fitness and overall improved health.

Additionally, all participants will be asked to donate 1.0 unit of blood to the Australian Red Cross. As a blood donor you will be recognised for your voluntary achievements and become part of a community. For many blood donors you become a lifeline; currently 1 in 3 Australians need blood. One single donation, when separated into its components, can help at least 3 different people.

Possible Risks

All exercise sessions will require a high level of effort from the participants. In rare circumstances it is possible that individuals could become light-headed and/or nauseous after completing such efforts. It is important to note that if you do become light headed and/or nauseous, you will not be allowed to leave the facility until the researcher is satisfied that you are fit to leave, or an emergency contact has been called to collect you. Nevertheless, we do not expect this to be an issue as all efforts are participant dependent; thus, no efforts will be in excess of a participant's own ability.

It is possible that during the collection of venous blood samples (at Murdoch University or the Australian Red Cross), participants could experience discomfort and potential bruising at the site of collection. Any discomfort is expected to not be greater than previous venous blood samples a participant may have provided for past medical reasons (i.e. standard pathology blood test). Nevertheless, if a participant does experience pain from this procedure the researcher will immediately attend to the area with compression and ice.

Distribution of Results

All data will be stored and analysed in a de-identifiable manner as such, no personal results will be provided to participants with the exception of aerobic fitness intensities and maximal power. The results of this study will be made publicly available through scientific manuscripts and through a brief description posted on the School of Psychology and Exercise Science website within three months of full data collection. In both instances, only average data will be used and no individual data will be presented.

Alternative Uses of Data

All data collected during this study (including blood samples) will be stored in a de-identifiable manner for the duration of five years at which time data will be destroyed or electronically erased as necessary. During this period (5 years), data collected from this study may

be used for additional scientific analysis related to this study. Furthermore, a small (500µl) sample of your blood will be retained for future metabolomics purposes. This blood will be used to help with method development specifically aimed at enhancing the precision of existing techniques.

Questions

If you would like to discuss any aspect of this study please feel free to contact Mr Nathan Lawler at n.lawler@murdoch.edu.au by phone at 9360 1450 or his primary supervisor Dr. Jeremiah Peiffer at 9360 7603. We would be happy to discuss any aspect of the research with you. You are welcome to contact us at any time to discuss any issue relating to the research study.

We would like to thank you in advance for your assistance with this research project. We look forward to hearing from you soon.

Sincerely,

Mr Nathan Lawler

This study has been approved by the Murdoch University Human Research Ethics Committee (xxxx/xx). If you have any reservation or complaint about the ethical conduct of this research, and wish to talk with an independent person, you may contact Murdoch University's Research Ethics Office (Tel. 08 9360 6677 (for overseas studies, +61 8 9360 6677) or e-mail ethics@murdoch.edu.au). Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

Appendix E Informed Consent Study 2

Mr. Andrew Govus
School of Exercise & Health Sciences
Edith Cowan University
270 Joondalup Drive
Joondalup WA, 6027
Tel: (08) 6304 3821, (02) 6214 7344
Mob: 0417094922
Email: a.govus@ecu.edu.au

The Effect of 14 Days of Live High, Train Low Altitude Exposure on Post-Exercise Iron Status in Well-Trained Endurance Runners

Informed Consent

I confirm the following:

- I have been provided with a copy of the information letter, explaining the research study
- I have read and understood the information provided
- I have been given the opportunity to ask questions and I have had any questions answered to my satisfaction
- I am aware that if I have any additional questions I can contact the research team

I understand that participation in the research project will involve:

Pre-Camp Phase (4 days)

- Measurement of iron profile (1 x venepuncture)
- Measurement of maximal oxygen consumption (VO_{2max}) (1 x 1 h session)
- Measurement of haemoglobin mass (1 x 15 min session)
- Standardised interval exercise sessions (2 x 1 h 30 min sessions, scheduled one day apart; 4 x venepunctures)
- Oral iron supplementation (1 x 325 mg ferrous sulphate & 562.4 ascorbic acid per day) for 14 days

14 Day Live High, Train Low Altitude Training Camp (2 weeks)

- Simulated altitude stimulus (14 days, 14 hours/day)
- Assessment of haemoglobin mass (1 x 15 min session)
- Standardised interval exercise sessions (2 x 1 h 30 min sessions, 1 day apart; 4 x venepunctures in total)
- Interim iron check (1 x venepuncture)
- Oral iron supplementation (1 x 325 mg ferrous sulphate & 562.4 ascorbic acid per day) for 14 days

Post Camp (1 day)

- Assessment of maximal oxygen consumption (1 x 1 h session)
- Assessment of haemoglobin mass (1 x 15 min session)

The ethical aspects of this study have been approved by the Edith Cowan University Human Research Ethics Committee. If you have any complaints or reservations about any ethical aspect of your participation in this research, you may contact the Human Research Ethics Committee through the Executive Office (Phone: +61 (08) 6304 2170). Any complaint you make will be treated in confidence, independently investigated and you will be informed of the outcome.

I understand that my information provided will be kept confidential, and that my identity will not be disclosed without consent

I understand that the information provided will only be used for the purposes of this research project and for future research in the area of sports science, and I understand how the information is to be used

I understand that I am free to withdraw from further participation at any time, without explanation or penalty

I freely agree to participate in the project.

I understand that other present or future researchers may use data collected during this study for research purposes other than those outlined in the information letter.

SIGNATURES

_____	_____
Participant	Date
_____	_____
Researcher	Date

The ethical aspects of this study have been approved by the Edith Cowan University Human Research Ethics Committee. If you have any complaints or reservations about any ethical aspect of your participation in this research, you may contact the Human Research Ethics Committee through the Executive Office (Phone: +61 (08) 6304 2170). Any complaint you make will be treated in confidence, independently investigated and you will be informed of the outcome.

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Appendix F Information Letter: Study 2

Mr. Andrew Govus
School of Exercise and Health Sciences
Edith Cowan University
270 Joondalup Drive
Joondalup WA, 6027
Tel: (08) 6304 3821
Mob: 0417094922
Email: a.govus@ecu.edu.au

The Effect of 14 Days of Live High, Train Low Altitude Exposure on Post-Exercise Iron Status in Well-Trained Endurance Runners

Information to Participants

PURPOSE

The purpose of this study is to investigate the influence of fourteen days altitude exposure on the post-exercise iron status in endurance runners.

Altitude training is frequently used by elite endurance runners to improve the oxygen carrying capacity of the blood. However, an increase in red blood cell production in response to hypoxia places a large demand on the body's iron stores, therefore, maintaining a healthy iron status at altitude is challenging and the risk of developing an iron deficiency is increased. To date, little is known about how iron balance is regulated at altitude. Hepcidin is a hormone produced by the liver responsible for regulating dietary iron absorption. In high concentrations, hepcidin reduces iron absorption and recycling. Recently, hepcidin levels have been shown to increase during the post-exercise recovery period in normoxic (sea-level) conditions, however, the post-exercise hepcidin response at altitude is currently unknown. Therefore, a greater understanding of the post-exercise hepcidin response at altitude may enable the development of new protocols to optimise iron absorption and refine iron supplementation practices for athletes living, training or competing at altitude, possibly resulting in an enhancement of the haematological benefits associated with altitude training and a reduction likelihood of developing an iron deficiency.

PROCEDURES

Should you choose to participate, you will be required to live at the AIS in Canberra for 18 days (4 day pre-testing and 14 days altitude exposure). The first four days will include a pre-camp assessment of iron status, aerobic capacity (VO_{2max}) and haemoglobin mass. For the following two weeks (14 days) you will participate in a Live High, Train Low altitude training camp, where we will investigate how you maintain iron balance after exercise during chronic altitude exposure.

Pre-Camp Phase (4 days)

1. *Assessment of Aerobic Capacity (1 x 45 min session)*

The first session will consist of a graded exercise test (GXT) to assess your maximal oxygen consumption (VO_{2max}) and determine your running velocity attained at VO_{2max} (vVO_{2max}). The GXT will be performed on a motorised treadmill where you will be required to run a number of 4 min workloads separated by a 1 min rest. The initial running speed will be 12 $km.h^{-1}$, with a 1 or 2 $km.h^{-1}$ increase until you reach volitional exhaustion. The estimated time commitment required for the GXT is 1 h.

2. *Assessment of Haemoglobin Mass (1 x 15 min session)*

During the second session we will determine your haemoglobin mass. This will involve breathing a small amount (1.2 $mL.kg^{-1}$) of carbon monoxide gas to determine your haemoglobin mass. The amount of carbon monoxide inhaled is small (the equivalent to one cigarette) and the test will be conducted used standardised protocols by a trained sport scientist.

3. *Standardised Interval Exercise Session (2 x 1 h 30 min sessions, scheduled one day apart)*

The four days will be a pre-camp phase, designed to assess how you regulate iron status after exercise at sea-level. During this time, you will maintain your regular training programme, however, once per week, you will perform a standardised interval exercise session (6 x 1000 m at 90% vVO_{2max}) to determine how you regulate iron levels after exercise at sea-level. Venous blood samples will be collected by a trained phlebotomist using standardised protocols via venepuncture of the forearm antecubital vein. Venous blood samples will be collected at two time points during each exercise session (pre-exercise and 3 h post-exercise).

Therefore, four (n=4) venepunctures will be performed throughout the four day pre-camp phase.

14 day Live High, Train Low Altitude Training Camp (2 weeks)

Simulated Altitude Stimulus (14 days, 14 hours/day)

During the 14 day live high, train low altitude camp, you will live in a simulated altitude chamber for 14 hours/day and breathe air with a reduced oxygen fraction (~15% oxygen) corresponding to an altitude of 3000 m.

Standardised Interval Exercise Sessions (4 x 1 h 30 min sessions, 1 week apart)

During this time, you will maintain your regular training programme, however, you will perform one standardised interval exercise session (6 x 1000 m at 90% vVO_{2max}) on a synthetic running track in normoxia and one standardised interval session in hypoxia (6 x 1000 m at 90% vVO_{2max}) to determine how you regulate iron levels after

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exercise in response to chronic altitude exposure. Venous blood samples will be collected at tow time points during each exercise session (pre-exercise & 3 h post-exercise).

Therefore, eight (n=8) venepunctures will be performed throughout the 14 day altitude camp. In addition, blood will also be collected 48 h after initial altitude exposure and at the conclusion of the camp to measure the effect of hypoxia on iron biomarkers. Hence, you will receive ten (n=10) venepuncture throughout the project.

Post-Camp Phase

Maximal oxygen consumption (VO_{2max}), haemoglobin mass and iron status will be measured after the 14-day altitude training camp as explained in the pre-camp phase.

Oral Iron Supplementation

To ensure you maintain sufficient iron stores when training at altitude you will be required to ingest an oral iron supplement (FerroGrad C, 325 mg ferrous sulphate & 562.4 ascorbic acid) daily before breakfast for the duration of the 14-day training camp.

RISKS

High Intensity Exercise

All exercise testing sessions require you to perform high intensity exercise. This type of exercise may be associated with discomfort and temporary muscle soreness post-exercise and/or injury.

Simulated Altitude Exposure

Risks of prolonged exposure to performing exercise in a hypoxic environment include breathlessness, nausea, headaches, dizziness and fatigue. These effects are temporary and often resolve once the hypoxic stimulus is removed. To ensure your safety, a trained sports physiologist will monitor the oxygen saturation of your blood during the first two nights of altitude exposure and during the hypoxic interval session via finger pulse oximetry.

CO Rebreathing

Carbon monoxide rebreathing is a safe technique used routinely to measure haemoglobin mass in medicine and sport science. The dose of carbon monoxide is small (approximately the same as one cigarette) and should not cause any long term health risks. You may experience some shortness of breath during the test, however, this resolves after the test. Additionally, carbon monoxide rebreathing may acutely impair aerobic performance for up to 3 h after the test.

Venous Blood Sampling

Venous blood samples are associated with a small risk of pain at the puncture site, bruising and infection. To minimise harm, a qualified phlebotomist using sterile equipment and standardised procedures will perform all venous blood procedures. A total of ten (n=10) venepunctures will be performed during this project.

BENEFITS

As part of the pre-participation screening procedures, we will determine your current iron status (serum ferritin levels), maximal oxygen consumption (VO_{2max}), anaerobic threshold. Furthermore, you will receive 14 days of altitude training and free food for 14 days.

CONFIDENTIALITY

The information collected in this study will be used to prepare a scientific report to be published in an academic journal. Your personal data will be stored securely and assigned an identification code, such that only those people directly involved in collecting information for the study will be able to recognise which person the information pertains to. We would also like to use the information collected during this study to be available to researchers associated with this study (co-investigators) for future analyses. However, please note that any information provided to other researchers will not contain any identifying details.

STORAGE OF BLOOD SAMPLES

All blood samples collected during this study will be stored in a -80 °C freezer at the Australian Institute of Sport (Canberra, ACT), Edith Cowan University (Perth, WA) or Murdoch University (Perth, WA), in a de-identified form for five years, after which time these data will be destroyed. During this time, employees of the biochemistry department at the Australian Institute of Sport, the principal investigator and specified co-investigators, will only be authorised to handle these blood specimens. Furthermore, within this five-year period, the co-investigators may re-analyse blood samples for additional scientific purposes related to this study. As such, a small (500 µl) sample of your blood will be retained for future analyses.

ALTERNATE USE OF DATA

Prior to analysis, all data will be de-identified to conceal your identity. The outcomes of these data will be presented reported in the scientific literature as a manuscript published in an academic journal as mean average data. All data obtained during this study may be re-analysed for future research purposes by the co-investigators. Electronic data obtained during this project will be stored indefinitely on an external hard drive, which will be kept in the possession of the chief investigator for a minimum of five years. Handwritten data recording sheets will be kept in a lockable filing cabinet at Edith Cowan University for a minimum of five years and accessible only by the chief investigator and co-investigators. At the conclusion of this project, you will be provided with a written report explaining your haematological and exercise test results. All questions about these results should be directed to the chief investigator, Mr. Andrew Govus (a.govus@ecu.edu.au).

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VOLUNTARY PARTICIPATION

Participation in this research is voluntary and you are free to withdraw at any time without prejudice. You can withdraw for any reason and you do not need to justify your decision. If you choose to withdraw from this study, and you are an employee or student at Edith Cowan University (ECU) this will not prejudice your right to compensatory under statutory or common law. With your permission, we may wish to retain the data that we have recorded from you during testing; otherwise, your records will be destroyed. This research project has been approved by the Human Research Ethics Committee, Edith Cowan University. If you have any questions relating to any of the information provided, please contact:

Students

Andrew Govus, School of Exercise & Health Science, Edith Cowan University, Mob: 0417094922, Email: a.govus@ecu.edu.au

Nathan Lawler, School of Psychology & Exercise Science, Murdoch University, Mob: 0409357582, Email: n.lawler@murdoch.edu.au

Supervisors

Dr. Laura Garvican, Physiology Discipline, Australian Institute of Sport, Tel: (02) 6214 1714, Email: laura.garvican@ausport.gov.au

Dr. Chris Abbiss, School of Exercise & Health Science, Edith Cowan University, Tel: (08) 6304 5740, Email: c.abbiss@ecu.edu.au

Dr. Peter Peeling, School of Sport Science, Exercise & Health, University of Western Australia, Tel: 0410667532 Email: ppeeling@uwa.edu.au

Dr. Jeremiah Peiffer, School of Psychology & Exercise Science, Murdoch University, Tel: (08) 9360 7603, Email: j.peiffer@murdoch.edu.au

If you have any concerns about this research, or would like to speak to an independent advisor, please contact:

Research Ethics Officer
Human Research Ethics Committee
Edith Cowan University
100 Joondalup Drive
Joondalup, WA, 6027
Phone: (08) 6304 2170
Email: research.ethics@ecu.edu.au

Thank You for Your Consideration

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Appendix G Example of Diet Diary and Training Diary

RECORDING YOUR DIET

Instructions For Using Easy Diet Diary

Firstly, download the free iPhone app – Easy Diet Diary

Add your personal details by touching the menu button in top left-hand corner

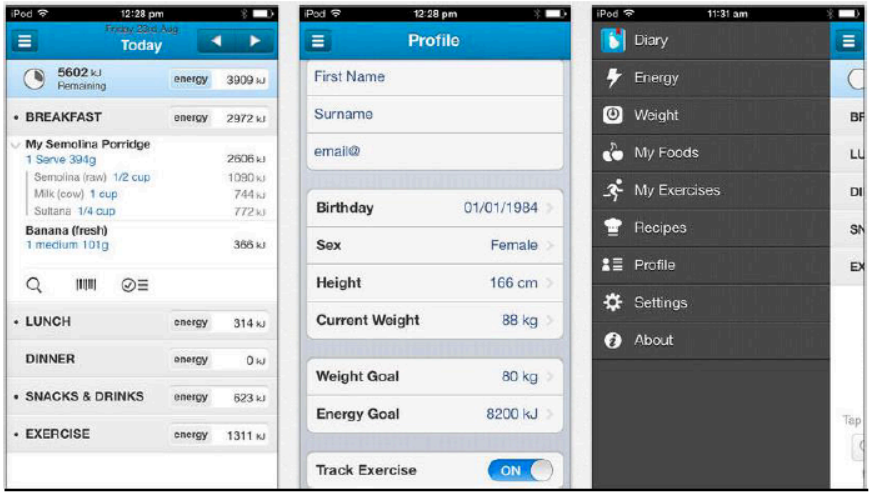
1. Select Profile
2. Fill in all fields
3. Select 'Track exercise"

Easy Diet Diary contains more than 50,000 Australian foods in its database. Food labels can be added by either manually searching for a food product, or by simply scanning a product barcode. If you wish you can add your own custom foods and recipes.

At the end of each week, email your diary through to Nathan
Touch the menu button in the top left-hand corner.

1. Select settings
2. Select 'email to your dietician'
3. Send to n.lawler@murdoch.edu.au

To ensure consistency between your testing sessions, you will need to provide information regarding your food and fluid intake for each day prior to performance testing.



If you do not have an iPhone or prefer the traditional way of pen and paper, you can record your food in a diary attached or it can be emailed to you.

To ensure consistency, we'll need you to provide information to us about your diet by filling out the attached logs. We ask that you keep your diet routine consistent across all experimental testing sessions. We ask you to eat similar foods the 24 hours prior to VO_{2max} and MMP_{4min} tests.

An example of a food log is:

Meal	*Time*	*Amount*	*Food*
Snack	6:15am	1	Chocolate mint ProMax bar
Breakfast	7:30am	1 Cups	Daily Juice Company orange juice
		2	Eggs – hard boiled
		2 pieces	Abbotts Wholegrain bread
Lunch	11:30am	12"	Subway turkey & cheese sub – with mayo

Keep your diet as normal as possible, as this will increase the accuracy when we analyse your diet. Brand names are helpful but not absolutely necessary. To enable us to give you the most accurate nutritional analysis following the study, a good idea is to bring in a cut out of the nutrient label – or take a picture. Try to enter your meals and snacks soon after you consume them so that you do not forget something. You do not need to measure your foods; approximating the amount is sufficient.

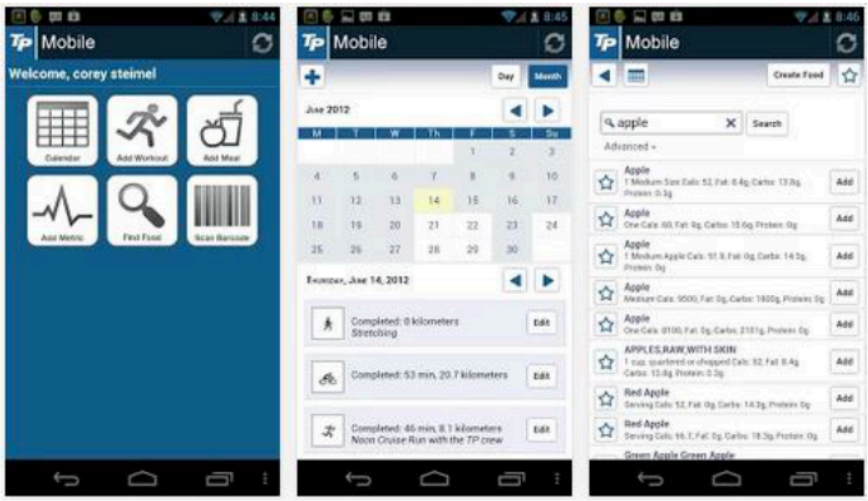
Instructions For Using Training Peaks

Firstly download the free android app – TrainingPeaks

Some of the great features of the Android App:

- Integrated Calendar gives you the most up to date information from your account
- Barcode scanner for food labels
- Fast, simple interface gives you exactly what you need to log on the go

TrainingPeaks Athlete Edition makes it simple to track all your training, nutrition and metrics like weight and sleep with charts to monitor your fitness and performance trends as they change over time.



MEAL	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
Pre-training food							
AM Training session							
Breakfast							
Morning Snack							
Lunch							
Afternoon snack							
PM Training Session							
Dinner							
Late Snack							
Thoughts & comments							

Appendix H Adult Pre-Exercise Screening Tool

ADULT PRE-EXERCISE SCREENING TOOL

This screening tool does not provide advice on a particular matter, nor does it substitute for advice from an appropriately qualified medical professional. No warranty of safety should result from its use. The screening system in no way guarantees against injury or death. No responsibility or liability whatsoever can be accepted by Exercise and Sports Science Australia, Fitness Australia or Sports Medicine Australia for any loss, damage or injury that may arise from any person acting on any statement or information contained in this tool.

Name: _____

Date of Birth: _____ Male ☐ Female ☐ Date: _____

STAGE 1 (COMPULSORY)

AIM: to identify those individuals with a known disease, or signs or symptoms of disease, who may be at a higher risk of an adverse event during physical activity/exercise. This stage is self administered and self evaluated.

Please circle response

1.	Has your doctor ever told you that you have a heart condition or have you ever suffered a stroke?	Yes	No
2.	Do you ever experience unexplained pains in your chest at rest or during physical activity/exercise?	Yes	No
3.	Do you ever feel faint or have spells of dizziness during physical activity/exercise that causes you to lose balance?	Yes	No
4.	Have you had an asthma attack requiring immediate medical attention at any time over the last 12 months?	Yes	No
5.	If you have diabetes (type I or type II) have you had trouble controlling your blood glucose in the last 3 months?	Yes	No
6.	Do you have any diagnosed muscle, bone or joint problems that you have been told could be made worse by participating in physical activity/exercise?	Yes	No
7.	Do you have any other medical condition(s) that may make it dangerous for you to participate in physical activity/exercise?	Yes	No

IF YOU ANSWERED 'YES' to any of the 7 questions, please seek guidance from your GP or appropriate allied health professional prior to undertaking physical activity/exercise

IF YOU ANSWERED 'NO' to all of the 7 questions, and you have no other concerns about your health, you may proceed to undertake light-moderate intensity physical activity/exercise

I believe that to the best of my knowledge, all of the information I have supplied within this tool is correct.

Signature _____ Date _____

ESSA EXERCISE & SPORTS SCIENCE AUSTRALIA Fitness Australia THE HEALTH & FITNESS INDUSTRY ASSOCIATION SPORTS MEDICINE AUSTRALIA

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EXERCISE INTENSITY GUIDELINES

INTENSITY CATEGORY	HEART RATE MEASURES	PERCEIVED EXERTION MEASURES	DESCRIPTIVE MEASURES
SEDENTARY	< 40% HRmax	Very, very light RPE# < 1	• Activities that usually involve sitting or lying and that have little additional movement and a low energy requirement
LIGHT	40 to <55% HRmax	Very light to light RPE# 1-2	• An aerobic activity that does not cause a noticeable change in breathing rate • An intensity that can be sustained for at least 60 minutes
MODERATE	55 to <70% HRmax	Moderate to somewhat hard RPE# 3-4	• An aerobic activity that is able to be conducted whilst maintaining a conversation uninterrupted • An intensity that may last between 30 and 60 minutes
VIGOROUS	70 to <90% HRmax	Hard RPE# 5-6	• An aerobic activity in which a conversation generally cannot be maintained uninterrupted • An intensity that may last up to about 30 minutes
HIGH	≥ 90% HRmax	Very hard RPE# ≥ 7	• An intensity that generally cannot be sustained for longer than about 10 minutes

= Borg's Rating of Perceived Exertion (RPE) scale, category scale 0-10

ADULT PRE-EXERCISE SCREENING TOOL

STAGE 2 (OPTIONAL)

Name: _____

Date of Birth: _____ Date: _____

AIM: To identify those individuals with risk factors or other conditions to assist with appropriate exercise prescription. This stage is to be administered by a qualified exercise professional.

		RISK FACTORS
1. Age Gender	≥ 45yrs Males or ≥ 55yrs Females +1 risk factor	
2. Family history of heart disease (eg: stroke, heart attack) Relative / Age / Relative / Age ☐ Father ____ ☐ Mother ____ ☐ Brother ____ ☐ Sister ____ ☐ Son ____ ☐ Daughter ____	If male < 55yrs = +1 risk factor If female < 65yrs = +1 risk factor Maximum of 1 risk factor for this question	
3. Do you smoke cigarettes on a daily or weekly basis or have you quit smoking in the last 6 months? Yes No If currently smoking, how many per day or week?	If yes, (smoke regularly or given up within the past 6 months) = +1 risk factor	
4. Describe your current physical activity/exercise levels: Sedentary ☐ Light ☐ Moderate ☐ Vigorous ☐ Frequency (sessions per week) ____ ____ ____ Duration (minutes per week) ____ ____ ____	If physical activity level < 150 min/ week = +1 risk factor If physical activity level ≥ 150 min/ week = -1 risk factor (vigorous physical activity/ exercise weighted x 2)	
5. Please state your height (cm) weight (kg)	BMI = _____ BMI ≥ 30 kg/m² = +1 risk factor	
6. Have you been told that you have high blood pressure? Yes No	If yes, = +1 risk factor	
7. Have you been told that you have high cholesterol? Yes No	If yes, = +1 risk factor	
8. Have you been told that you have high blood sugar? Yes No	If yes, = +1 risk factor	

Note: Refer over page for risk stratification.

STAGE 2 Total Risk Factors =

9.	Have you spent time in hospital (including day admission) for any medical condition/illness/injury during the last 12 months? Yes No	If yes, provide details
10.	Are you currently taking a prescribed medication(s) for any medical conditions(s)? Yes No	If yes, what is the medical condition(s)?
11.	Are you pregnant or have you given birth within the last 12 months? Yes No	If yes, provide details. I am _____ months pregnant or postnatal (circle).
12.	Do you have any muscle, bone or joint pain or soreness that is made worse by particular types of activity? Yes No	If yes, provide details

STAGE 3 (OPTIONAL)

AIM: To obtain pre-exercise baseline measurements of other recognised cardiovascular and metabolic risk factors. This stage is to be administered by a qualified exercise professional. (Measures 1, 2 & 3 – minimum qualification, Certificate III in Fitness; Measures 4 and 5 minimum level, Exercise Physiologist*).

	RESULTS		RISK FACTORS
1. BMI (kg/m²)		BMI ≥ 30 kg/m² = +1 risk factor	
2. Waist girth (cm)		Waist > 94 cm for men and > 80 cm for women = +1 risk factor	
3. Resting BP (mmHg)		SBP ≥140 mmHg or DBP ≥90 mmHg = +1 risk factor	
4. Fasting lipid profile*		Total cholesterol ≥ 5.20 mmol/L = +1 risk factor	
Total cholesterol		HDL cholesterol >1.55 mmol/L = -1 risk factor	
HDL		HDL cholesterol < 1.00 mmol/L = +1 risk factor	
Triglycerides		Triglycerides ≥ 1.70 mmol/L = +1 risk factor	
LDL		LDL cholesterol ≥ 3.40 mmol/L = +1 risk factor	
5 Fasting blood glucose*		Fasting glucose ≥ 5.50 mmol = +1 risk factor	
		STAGE 3 Total Risk Factors =	

RISK STRATIFICATION

Total stage 2
or
Total stage 3
Plus stage 2 (Q1 - Q4)

≥ 2 RISK FACTORS – MODERATE RISK CLIENTS
Individuals at moderate risk may participate in aerobic physical activity/exercise at a light or moderate intensity (Refer to the exercise intensity table on page 2)

< 2 RISK FACTORS – LOW RISK CLIENTS
Individuals at low risk may participate in aerobic physical activity/exercise up to a vigorous or high intensity (Refer to the exercise intensity table on page 2)

Note: If stage 3 is completed, identified risk factors from stage 2 (Q1-4) and stage 3 should be combined to indicate risk. If there are extreme or multiple risk factors, the exercise professional should use professional judgement to decide whether further medical advice is required.

Appendix I Blood Donation Screening Tool

APPENDIX E

Thinking of Giving Blood?

Please review the following more common factors that will determine if you can or cannot give blood*

Most people are able to give blood if they;	
Feel fit and healthy	☐ Yes ☐ No
Are aged between 16 and 70	☐ Yes ☐ No
Weigh over 45kg (weigh over 50kg if under age 18)	☐ Yes ☐ No
You may be temporarily unable to give blood if you:	
Are on certain medications or antibiotics, including oral acne medication	☐ Yes ☐ No
Have a cold or feeling unwell in any way	☐ Yes ☐ No
Recently had surgery or major dental work	☐ Yes ☐ No
Recently had a piercing	☐ Yes ☐ No
Recently travelled to areas affected by Malaria (within the last 4 months), West Nile Virus (within the last 8 weeks) or Dengue Fever (within the last 4 weeks).	☐ Yes ☐ No
You will not be able to give blood if you:	
Have visited or lived in the UK for a cumulative total of 6 months or more between 1980 and 1996	☐ Yes ☐ No
Have engaged in male to male sexual activity or other specified at risk sexual activity in the past 12 months	☐ Yes ☐ No
Have recently been pregnant or given birth	☐ Yes ☐ No
Have had a tattoo in the past 6 months	☐ Yes ☐ No
Have ever had a serious heart condition	☐ Yes ☐ No
Have ever had a serious blood disorder or disease	☐ Yes ☐ No
Have ever "used drugs" by injection or been injected with drugs not prescribed by a doctor or dentist	☐ Yes ☐ No

Haemoglobin measure_____

Before giving blood:

1. Drink up – in the 24 hours before donation, especially in warm weather, and have at least 4 good-sized glasses of water/juice in the 3 hours prior to your donation
2. Eat up – make sure you eat something in the 3 hours before donating
3. Bring ID – please bring at least one form of photo identification

If you are unsure about your eligibility to give blood, please feel free to contact Mr. Nathan Lawler at n.lawler@murdoch.edu.au or by phone 0409 357 582

*Further eligibility criteria apply. You will be required to complete a comprehensive questionnaire before your eligibility to donate is determined.